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Peri-prosthetic interface tissue around aseptic loosened prostheses: not waste, but a potential therapeutic target?

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Peri-prosthetic interface tissue around aseptic loosened prostheses: not waste, but a potential therapeutic target?

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	Prof. Dr. J. Dankelman, TU Delft
	dr. P.K. Bos, Erasmus MC

Contents

1 INTRODUCTION

Clinical problem

Total joint replacement (TJR) is an effective surgical intervention for end-stage joint diseases as osteoarthritis and rheumatoid arthritis. Annually approximately 28.000 total hip (THA) and 24.000 total knee arthroplasties (TKA) are being performed in The Netherlands.[1] These numbers increased in the last 5 years and is expected to increase even more due to the rising incidence of obesity and a more active lifestyle of the elderly.[1, 2] The survival-rates of THA and TKA show consistent results with failure of only 5 -10% after 10 years and up to 20% of revisions at 20 years follow-up.[3-6] Particularly in younger, more active patients the long-term survival of TJRs is reduced compared to the elderly population (older than 65 years).[3-8] Revision surgery consists of removal of the loosened components and peri-prosthetic interface soft tissue, sometimes augmenting cortical and spongious bone loss with allograft bone, and subsequent insertion of new components. Large bone defects, caused by both osteolytic lesions as well as stress shielding create not only a technical surgical challenge to fixate new implant components, but may also cause intraoperative fractures during removal of the implant. Consequently, these revision THA and TKA surgeries are often highly demanding for the patient and can be associated with complications, hence creating new morbidity, particularly in elderly patients with a poor general health condition.[9-11] Additionally, the clinical and functional results of extensive revision arthroplasty surgery are less favourable compared to primary arthroplasty surgery.[12-14] Therefore, therapies less demanding than this extensive revision surgery or even prevention of extensive bone loss during the loosening process would improve quality of patient care.

Aseptic loosening

Aseptic loosening is reported as a major factor limiting the long-term survival of TJRs, accounting for about 50% of THA revisions and 30% of TKA revisions.[1, 15, 16] Aseptic loosening refers to a process during which stable and osseointegrated implants become loose as the bone surrounding the implant is resorbed. This process is regulated by a complex interaction between both biomechanical factors (i.e. stress shielding) and biological factors (i.e. response to wear debris particles through bone signalling at cellar levels).[17, 18] Particulate wear debris, continuously generated by articulating motion at the bearing surfaces, has been implicated as one of the primary causes initiating peri-prosthetic bone loss and implant loosening.[17, 19, 20] Wear debris can be phagocytized by various cell types, triggering a continuous localized peri-prosthetic inflammatory response through the production of inflammatory mediators. These inflammatory mediators create a microenvironment that favours osteoclast formation and subsequently peri-prosthetic bone resorption. The rate of peri-prosthetic bone loss may vary between patients due to differences in the properties and amount of particulate wear debris, different patterns of biomechanical failure of artificial implants and differences in the individual host immune response which can be related to an individual genotype.[21-27] Therefore, evaluation of individual biological responses is possibly essential to intervene with the process of aseptic loosening.

Peri-prosthetic interface tissue

During the process of aseptic loosening a loose connective fibrous-like tissue develops at the interface between an implant and the bone bed. This so-called peri-prosthetic interface tissue exhibits a heterogeneous cellular composition that generally includes monocyte/macrophage lineage cells (macrophages, foreign body giant cells, and osteoclasts), fibroblasts, endothelial cells, osteoblasts and lymphocytes.[28, 29] Most of these cell types are able to phagocytize wear debris particles and secrete a number of proteolytic enzymes as well as pro-inflammatory and osteoclastogenic cytokines. Proteolytic enzymes, such as matrix metalloproteinases (MMP's), can directly degrade demineralized collagen matrix.[30] The pro-inflammatory cytokines, such as tumor necrosis factor alpha (TNF-α) and interleukin 1 and 6 (IL-1, IL-6), provoke cellular proliferation, stimulate osteoclast activity and/or decrease osteoblast function and thereby disrupt the homeostasis of bone metabolism.[17, 20, 31] Bone metabolism is governed by a delicate balance between bone formation by osteoblasts and bone resorption by osteoclasts. This process is tightly regulated by local and endocrine factors. In normal bone metabolism there is a balance between levels of osteolytic and osteogenic cytokines. In aseptic loosening, this balance is disrupted resulting in a net bone loss around the implant.[32]

Most studies on peri-prosthetic interface tissue focus on the relation between the local production of cytokines and enzymes and their effect on the peri-prosthetic osteolytic process. Histological examination reveals a high inter- and intra-sample variation in both cellular and cytokine profiles within the peri-prosthetic interface tissue, which may represent different stages of loosening in different topological areas.[33-36] This heterogeneity is probably due to the variable biological, mechanical, and material microenvironments along the bone-implant interface.[33, 36-38] Histological evidence also indicates that peri-prosthetic interface tissue is not a tissue with solely bone "destructive" properties. Bone remodelling around the implant has been shown by the presence of intramembranous formation of osteoid, and the production of immature bone with poor quality.[39, 40] Furthermore, several cell types within the peri-prosthetic interface tissue have been shown to produce osteoblast specific proteins [41] as well as to exhibit an increased expression of several bone morphogenetic proteins (BMPs)[42], which are regulators and potent inducers of osteoblast differentiation.[43] The local increase of osteogenic proteins, BMPs and bone remodelling around the implant may indicate that osteogenesis also takes place in peri-prosthetic interface tissue.

Targets for treatment

Through the years, many efforts have been made at improving the quality of primary joint replacements and thereby reducing the prevalence of aseptic loosening and the potential need for revision surgery. For example, alternative bearing surfaces have been developed which significant reduced the amount of wear.[44-47] However, regardless of these efforts, aseptic loosening still persists. Therefore continued research into new therapies to treat aseptic loosening is necessary to prolong the lifetime of prostheses. So far, studies aiming at identifying targets for treatment of aseptic loosening have primarily focused on interfering with the osteolytic process. However, only partial inhibition of bone resorption could be achieved in studies using non-steroidal anti-inflammatory drugs (NSAIDs) and/or antibodies to specific osteolytic mediators.[48-50] The results of the clinical use of bisphosphonates to treat bone resorption in aseptic loosening were inconsistent.[51, 52] Alternatively, therapeutic agents targeted at improving bone formation in the peri-prosthetic osteolytic areas are also likely to countermeasure the osteolytic process. However, hardly any studies exist on this topic. In fact, the role of peri-prosthetic interface tissue cells in bone formation is yet even unclear.

Outline of this thesis

The objective of the research described in this thesis is to increase the knowledge on the biology behind the process of aseptic loosening. For this purpose, we aim to study the loosening process from three different biological perspectives, according to the following research questions:

1. Does the cellular content of peri-prosthetic interface tissue shed new light on the mechanism of implant loosening?
2. Do peri-prosthetic interface tissue cells possess osteogenic potential, which can ultimately be used to prevent or slow loosening?
3. Does the individual host immune response relate to prosthesis migration, which can ultimately predict loosening?

The first research question is addressed in **Chapters 2** and **3**. In **Chapter 2**, an overview of currently known cellular mechanisms involved in aseptic loosening, based on *in vitro* findings, is given. The cellular mechanisms are further explored in **Chapter 3**, where the cellular characteristics of peri-prosthetic interface tissue samples are studied by determining cell-specific gene expression patterns and using immunohistochemistry. In **Chapters 4** and **5**, the second research question is addressed. In **Chapter 4**, the possibility to enhance bone regeneration, by intervening with signalling pathways which are important for osteogenic differentiation, is studied in human and murine cell lines. Results from this study are used in

Chapter 5, where we investigate whether cells derived from peri-prosthetic interface tissue are capable of differentiation into the osteoblastic lineage. The third research question is addressed in **Chapter 6**, where we investigate the relation between non-specific cytokine (innate immune) responses and the early migration of prostheses. Finally, **Chapter 7** concludes this thesis with a summary and general discussion including future directives on the treatment of aseptic loosening.

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CELLULAR MECHANISMS INVOLVED IN ASEPTIC PROSTHESIS LOOSENING: A DESCRIPTIVE SYSTEMATIC REVIEW OF *IN VITRO* STUDIES

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2

Abstract

Background

Aseptic loosening is the most common long-term cause of implant failure, but its underlying biological mechanism is complex and still not elucidated completely. Therefore, this review aims to clarify the characteristics of peri-prosthetic tissue based on *in vitro* findings in order to provide an overview of the currently proposed cellular mechanisms involved in implant loosening.

Methods

A systematic search in various databases revealed 51 eligible studies describing *in vitro* findings on peri-prosthetic fibrous tissue obtained from aseptic loosened implants. Besides general study characteristics, the following outcome measures were extracted: production of biochemical factors, response to particles, osteoclastogenic capacity, and osteogenic capacity.

Results

Both macrophages and fibroblasts seem to be actively involved in osteoclastogenesis and pathologic bone resorption through production of inflammatory cytokines, chemokines, matrix degrading enzymes, osteoclastogenic factors and angiogenic factors. Particles, particularly titanium particles, interfered with all these factors. No papers reported on the osteogenic capacity of peri-prosthetic tissue. However, the tissue was shown to produce factors that suppress osteoblast function, indicating that (effects on) osteoblasts do play a role in the process of loosening.

Conclusion

This literature study shows that the role of fibroblasts and osteoblasts in aseptic loosening is underestimated and that these cells could be potential targets for treatment. However, high variability in all reported outcome measures frequently hampered interpretation of the results, which underlines the need for a more uniform and in-depth description of patient-, prosthesis-, and tissue-related characteristics in future studies in order to address the mechanism of aseptic loosening and its potential therapeutic targets more effectively.

Keywords: Peri-prosthetic osteolysis; Total joint replacement; Interface tissue; Cell culture

Introduction

Aseptic loosening is the most common long-term cause of failure in total hip and knee arthroplasties, accounting for approximately 70% of revision surgeries after hip replacement.[1] Peri-prosthetic loosening is a process in which the once firm bond between prosthesis and bone or cement is lost.[1] A fibrous loosening membrane with poor mechanical properties is formed, triggering bone resorption and prosthesis migration. A complex interplay between mechanical and biological factors is known to elicit the loosening process.[2] Particulate wear debris has been implicated as one of the primary causes initiating peri-prosthetic bone loss and implant loosening. Wear can be phagocytized by various cell types of which the most important cellular target is believed to be the macrophage.[3] Phagocytosis of wear triggers the immune system causing production of inflammatory mediators that are implicated in osteoclast formation and activation. As such, the final cellular consequence in the action of wear is an excess of osteoclast activity, which results in disturbed bone remodelling and ultimately osteolysis.

Although many reports have been published on the pathogenesis of loosening, the precise biological mechanisms underlying this process have still not yet been elucidated completely. In a recent review on histological and immunological aspects of aseptic loosening, it was concluded that further investigation of peri-prosthetic tissues in terms of target cells, pathways and proteins is required.[4] In that review, however, studies based on other techniques than histology were excluded and thus potentially important cellular and molecular mechanisms may have been missed.

Therefore, this review aims to clarify the characteristics of peri-prosthetic tissue based on *in vitro* findings in order to provide an overview of the currently proposed cellular mechanisms involved in implant loosening. Specifically, we aimed to assess the (1) production of biochemical factors, (2) response to particles, (3) osteoclastogenic capacity, and (4) osteogenic capacity of peri-prosthetic tissue *in vitro*.

Search criteria and strategy

Search strategy

A thorough search strategy (see Supplementary data S-1) was composed in collaboration with an experienced information specialist (JWS). The following databases were searched up to April 2014: PubMed, Medline, Embase, Web of Science, Cochrane Library, CENTRAL, CINAHL, Academic Search Premier, and ScienceDirect. The search strategy consisted of the following components, each defined by a combination of controlled vocabulary and free text terms: 1) Interface / peri-prosthetic / granulation (synovial) tissue OR interface / peri-prosthetic / granulation (pseudo) membrane; 2) Cell culture / organ culture / tissue culture / in vitro / in situ hybridization / polymerase chain reaction / western blotting; and

3) Arthroplasty / (aseptic) loosening / osteolysis / hip OR knee prosthesis / hip OR knee replacement. The addition of the specific search terms 'in situ hybridization, polymerase chain reaction and western blotting' was necessary as known potentially relevant papers turned out to be missing in the search in case only direct cell culture related terms were used. Additionally, three trial register sites were searched: the WHO International Clinical Trials Registry Platform, ClinicalTrials.gov and the ISRCTN registry. There were no initial restrictions on language and date.

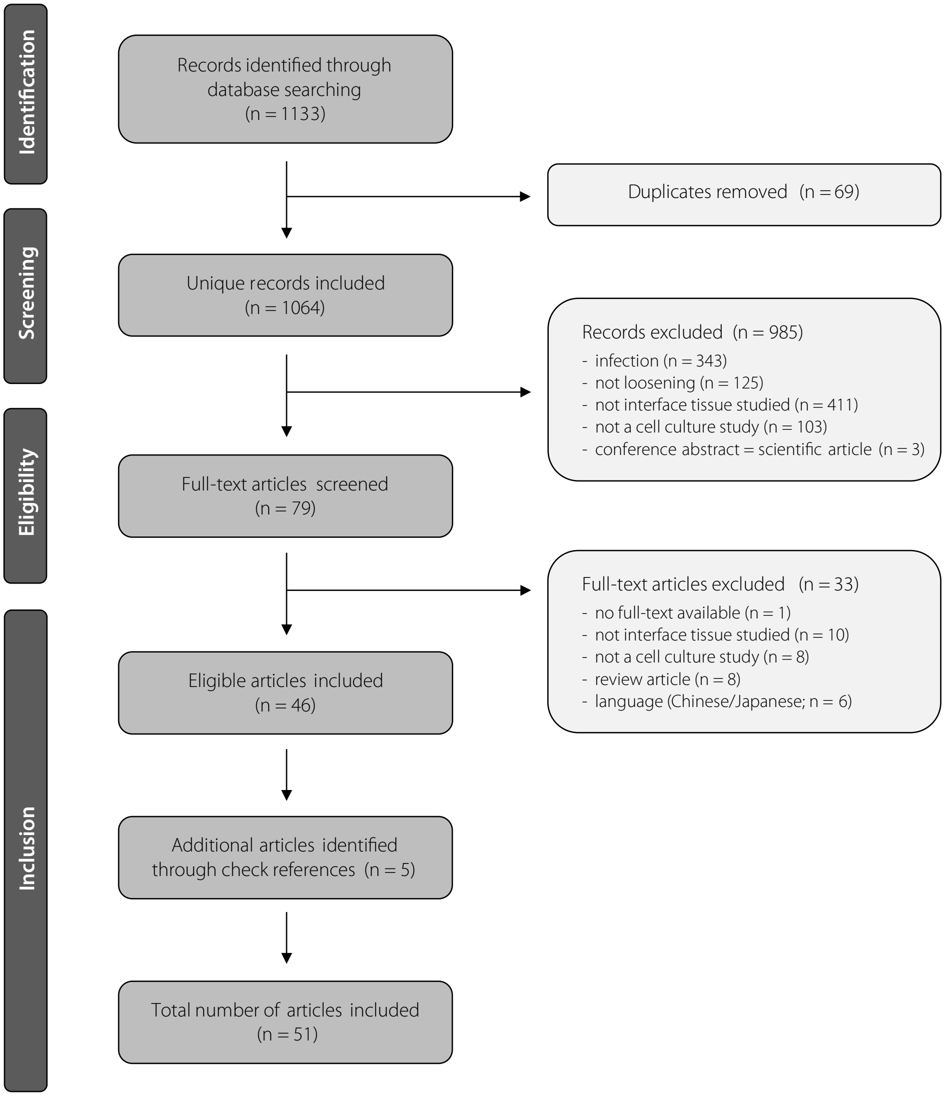

Figure 1: PRISMA flow diagram of screening and selection process.

Screening and inclusion

A flow diagram of the screening and inclusion process is shown in Figure 1. Eleven hundred thirty-three records identified through the electronic searches were collected in an electronic reference database. After removal of duplicates, 1064 unique records remained. Based on title and abstract, two reviewers (AEO, MAES) independently screened scientific articles, conference abstracts and reports from trial registers using the following inclusion criteria: 1) the study had to deal with aseptic loosening; 2) the studied material had to be peri-prosthetic fibrous tissue; and 3) the outcome measurement(s) had to include particular culture of peri-prosthetic tissue or analysis of material obtained during culturing the tissue (e.g. RNA or conditioned culture medium). A record was considered eligible when it met all three inclusion criteria. In case the title and/or abstract were inconclusive, the record remained eligible. Findings of both reviewers were compared and any disagreements were resolved by consensus, resulting in the exclusion of 985 records. The full-text of the remaining 79 eligible records were evaluated by AEO and included when the above-mentioned inclusion criteria were met. In case of any doubt regarding the eligibility of a paper, the paper was evaluated by MAES as well and agreement on eligibility was achieved by consensus. Another 33 records were excluded. References of the 46 included articles were checked for potentially eligible studies that were not identified with the original search strategy in order to minimize the risk of missing relevant studies, which resulted in the additional inclusion of 5 papers. Finally, 51 papers were included for data extraction.

Data extraction

From the included papers, data were extracted systematically and collected in a pre-defined electronic document, the so-called data-extraction form. Recorded article information included year of publication, authors (first, last), study group, and running title. With respect to the patient characteristics, the number of subjects in the study, sociodemographic data of the subjects (gender, age), type of surgery (THA, TKA, other), indication for surgery (OA, RA, other), time to revision, and presence of a control group (yes/no; if yes, number, gender, age) were extracted. Regarding the study characteristics, the study aim (primary, secondary), inclusion and exclusion criteria, type of prosthesis fixation (cemented, cementless), prosthesis material, source of study material (human, animal), type of study material (tissue, conditioned medium, other), tissue/cell culture-related parameters (studied cell type(s), passage number used for culture experiment(s), experimental culture conditions), and type of methods used besides cell culture (immunohistochemistry, PCR, FACS, ELISA, other) were recorded. With respect to the study outcomes, the following data were extracted: production of biochemical factors, response to particles, osteoclastogenic capacity, and osteogenic capacity.

The search included 7 papers on animal studies (sheep [5], dog [6, 7], rabbit [8-10] and rat [11]), in which animals underwent either spinal, hip or knee surgery. Since findings were similar to those found in the human studies, these papers will not be discussed in this review. Categorizing the 44 human studies in the predefined study outcomes of interest revealed 30 papers describing the production of biochemical factors by cells from the peri-prosthetic fibrous tissue and 7 papers in which response to particles was determined. The osteoclastogenic capacity of cells from the fibrous tissue was explored in 18 papers, whereas no papers on the osteogenic capacity of fibrous tissue cells were found. Two papers [12, 13] presented data on *in vitro* fibrous tissue characteristics other than our predefined study outcomes of interest and as such are not further discussed. Data were synthesized in descriptive and tabular format because of the heterogeneity of the study outcomes and are presented for each study outcome category separately.

Results

Production of biochemical factors

Table 1 summarizes the findings of the 29 papers focusing on the production of biochemical factors by cells from the peri-prosthetic fibrous tissue (one paper reported on production of biochemical factors in response to particles solely and is therefore mentioned in the section 'Response to particles'). Biochemical factors were generally determined in conditioned medium from tissue cultures, with 2 papers showing that macrophages were the main cell responsible for the measured factors.[14, 15] In 7 papers fibrous tissue fibroblasts were used specifically [16-22] and in 1 paper lymphocytes isolated from the fibrous tissue were used.[23] Inflammatory mediator production was measured in 19 papers [14-19, 21, 23-34], production of matrix molecules/enzymes in 3 papers [20, 22, 35], and 7 papers reported on both.[36-42]

PGE_2, IL-1, IL-6 and TNFα were the most common inflammatory mediators measured. The extent of osteolysis was reported to correlate with levels of these mediators.[39] Findings on comparisons made between cemented and cementless samples varied, however, tissues from cementless prostheses more often tended to produce the highest mediator levels.[28, 37, 39] One study compared mediator production between hip and knee samples and showed tissue from failed hip prostheses to be more active than tissue from failed knee prostheses.[39] Another study compared mediator production between samples from linear and erosive bone loss and found higher levels in the case of linear bone loss.[30] A study in which production of PGE_2 was compared between deep, intermediate and superficial layers of capsule tissue, showed the deeper layer to produce the highest PGE_2 levels.[27] Four papers [15-17, 32] described the production of RANK/RANKL and/or m-CSF and observed endogenous production of these mediators. The production seemed unaffected by inflammatory cytokines, but could be enhanced by particles.

Table 1: Production of biochemical factors by peri-prosthetic tissue cells

Article - first author - last author - year	**Patients** - number (m/f) - age (yrs)* - primary diagnosis (n) - time to revision (yrs)*	**Prosthesis** - type (n) - fixation (n) - material	**Study material** - IFT tissue/cells - CM - bone cells - immune cells	**Study outcome – Cytokine/Matrix production** Cytokines / Chemokines	 Matrix molecules / Enzymes
Goldring Harris 1983 [12]	20 (NR) NR NR NR	THA C NR	+ + - -	PGE2 (both in organ and cell culture): Membranes > capsules Membranes = RA synovium	Collagenase (both in organ and cell culture)
Goodman Masada 1989 [13]	16 (NR) NR NR NR	THA C (14), NC (2) NR	+ + - -	High PGE2, no IL-1β, with PGE2: Loose > non-loose membrane cultures	Low collagenase in both loose and non-loose membrane cultures
Appel Herman 1990 [3]	30 (16/14) 60 (22 – 83) OA 7 (2 – 15)	THA C NR	+ + - -	IL-1, TNFα and PGE2: membranes = synovial tissue samples	
Kim McClain 1993 [20]	34 (15/19) 57 OA (17), other (17) 5	THA C (14), NC (20) CoCr, Ti, SS	+ + - -	PGE2 and IL-1α: Membranes > fibrous pseudocapsules	Gelatinase and collagenase: Membranes > fibrous pseudocapsules
Glant Kuettner 1994 [10]	11 (5/6) NR NR NR	THA C (4), NC (7) NR	+ + - -	IL-1α, IL-1β and IL-6 over culture time, with IL-1α > IL-1β	
Horikoshi Rubash 1994 [16]	52 (18/34) 70 OA (37), other (15) 6	THA (36), TKA (16) C (26), NC (26) CoCr, Ti, SS	+ + - -	PGE2, IL-1α, IL-1β, IL-6 and TNFα: Membranes > fibrous pseudocapsules Hips > knees PGE2: cemented > non-cemented TNFα and IL-6: non-cemented > cemented	Gelatinase and collagenase: Membranes > fibrous pseudocapsules Knees > hips Stromelysin: Membranes > fibrous pseudocapsules Hips > knees

Table 1: Production of biochemical factors by peri-prosthetic tissue cells (*Continued*)

Article - first author - last author - year	**Patients** - number (m/f) - age (yrs)* - primary diagnosis (n) - time to revision (yrs)*	**Prosthesis** - type (n) - fixation (n) - material	**Study material** - IFT tissue/cells - CM - bone cells - immune cells	**Study outcome – Cytokine/Matrix production** Cytokines / Chemokines	 Matrix molecules / Enzymes
Kim Rubash 1994 [19]	63 (25/38) 61 OA (27), other (36) 6	THA NC CoCr, Ti	+ + - -	PGE2: Cr = Ti = non-PE With osteolysis = without osteolysis IL-1α: Cr and Ti, but absent in non-PE	Collagenase: Cr and Ti > non-PE With osteolysis = without osteolysis
Ohlin Lerner 1994 [31]	5 (1/4) 67 OA (4), other (1) 13	THA NR Delrin◊, PE	+ + - -	PGE2: deeper layer > superficial and intermediate	
Perry Atkins 1995 [35]	52 (NR) 75 (44 – 85) OA (46), RA (4), other (2) 11 (2 – 20)	THA (39), TKA (12), elbow (1) C NR	- + - -	IL-1β: Pseudocapsule > OA and RA synovia Correlation with $CD14^+$ and $CD68^+$ cells IL-6 and PGE2: Pseudocapsule = OA and RA synovia Correlation with $CD14^+$, $CD15^+$ and $CD45^+$ cells	
Yao Galante 1995 [57]	6 (5/1) 36 – 74 OA (4), other (2) 3 – 12	THA NR CoCr, Ti	+ + + -		Collagenase, stromelysin and TIMP: Membranes > fetal synovia

Table 1: Production of biochemical factors by peri-prosthetic tissue cells (*Continued*)

Article - first author - last author - year	**Patients** - number (m/f) - age (yrs)* - primary diagnosis (n) - time to revision (yrs)*	**Prosthesis** - type (n) - fixation (n) - material	**Study material** - IFT tissue/cells - CM - bone cells - immune cells	**Study outcome – Cytokine/Matrix production** Cytokines / Chemokines	 Matrix molecules / Enzymes
Shanbhag Glant 1995 [47]	26 (9/17) 58 NR 9	THA C (12), NC (14) CoCr, Ti	- + - -	PGE2, IL-6, IL-8 and TNFα: IFT < OA Cemented = non-cemented IL-1α: IFT > OA non-cemented > cemented	
Yokohama Okada 1995 [58]	34 (2/32) 72 (41 – 74) NR 8 (3 – 20)	THA C (15), NC (19) CoCr, Ti	+ + - -		(pro)MMP-9, active MMP-2 and gelatinase: Membranes > pseudocapsules Cemented > non-cemented MMP-1, (pro)MMP-2, TIMPs and collagenase: Membranes = pseudocapsules MMP-3: Membranes < pseudocapsules
Perry Elson 1996 [34]	40 (13/27) 74 (38 – 89) OA (32), RA (2), other (6) 11 (1 – 22)	THA (29), TKA (11) C (38), NC (2) NR	- + - -	IL-6, IL-1β, TNFα and PGE2: CMs with bone-resorbing activity > without	
Perry Learmonth 1997 [36]	39 (11/28) 74 OA (32), RA (2), other (5) 10	THA (28), TKA (11) C CoCr, Ti, SS	- + - -	IL-1β, IL-6, PGE2, TGFβ: linear implants > erosive implants TNFα: linear implants = erosive implants	

Table 1: Production of biochemical factors by peri-prosthetic tissue cells (*Continued*)

Article - first author - last author - year	**Patients** - number (m/f) - age (yrs)* - primary diagnosis (n) - time to revision (yrs)*	**Prosthesis** - type (n) - fixation (n) - material	**Study material** - IFT tissue/cells - CM - bone cells - immune cells	**Study outcome – Cytokine/Matrix production** Cytokines / Chemokines	 Matrix molecules / Enzymes
Moreschini Romanini 1997 [25]	9 (2/7) 62 NR 5	THA C (2), NC (7) CoCr, Ti, SS, PE	+ + - -	IL-1β (in 4 out of 9 samples) are present during begin and end of culture period	Hyaluronic acid during culturing Type III procollagen is high during entire culture period
Neale Athanasou 1999 [26]	9 (3/6) 74 NR 10	THA C (8), NC (1) CoCr, Ti, SS, PE	+ + + -	Co-culturing of IFT MQs and rat bone cells: mCSF, IL-1β and IL-6, but not TNFα	
Trindade Smith 2001 [53]	5 (NR) NR NR NR	NR NR NR	+ - - +	IL-6 and TNFα: Membrane lymphocytes > blood lymphocytes Co-culturing IFT MQs and lymphocytes did not enhance cytokine release	
Lavigne Fernandes 2002 [23]	20 (12/8) 68 (42 – 79) OA 58 (34 – 11)	THA NR Metal, PE	+ + - -	IL-1β, IL-6, TNFα and PGE2 ¯ by Tenidap Only PGE2 ¯ by Diclofenac	
Ito Shindo 2004 [17]	40 (11/29) 67 OA (30), RA (1), other (9) 14	THA C (24), NC (16) NR	+ + + -	High IL-6, low TNFα Addition of CM to human bone cells IL-6 IL-6 ¯ by anti-IL-1β, indomethacin and dexamethasone, but not by anti-TNFα *Remark: Cell culture only n =1*	

Table 1: Production of biochemical factors by peri-prosthetic tissue cells (*Continued*)

Article - first author - last author - year	**Patients** - number (m/f) - age (yrs)* - primary diagnosis (n) - time to revision (yrs)*	**Prosthesis** - type (n) - fixation (n) - material	**Study material** - IFT tissue/cells - CM - bone cells - immune cells	**Study outcome – Cytokine/Matrix production** Cytokines / Chemokines	 Matrix molecules / Enzymes
Mandelin Konttinen 2005 [24]	6 (4/2) 70 ± 11 OA NR	THA NR NR	+ + + -	OPG: Fibrous capsule < IFT < bone cells by TNFα in IFT RANKL: IFT = bone cells	
Koreny Glant 2006 [21]	32 (18/14) 62 (34 – 91) OA (NR), RA (NR) 10	THA (23), TKA (9) NR NR	+ + - +	IL-1β, IL-6, IL-8, TNFα, MCP-1 and VEGF: Membranes > normal synovial tissue OPG and RANKL present Addition of CM to IFT Fibs RANKL	
Shanbhag Rubash 2007 [48]	13 (5/8) 63 OA 17	THA C (5), NC (8) NR	+ + - -	IL-6, IL-8, IP-10, MIG, sICAM-1, MCP-1, sCD23, TGFβ, IL-1β, IL-2, IL-10 and G-CSF	
Tunyogi-Csapo Glant 2007 [54]	32 (18/14) 62 (34 – 91) OA (NR), RA (NR) 10	THA (23), TKA (9) NR NR	+ + - -	Addition of CM to IFT Fibs 3 VEGF isoforms	
Syggelos Panagiotopoulos 2007 [52]	10 (0/10) 67 (46 – 84) NR 12 (1 – 20)	THA (7), TKA (3) C (8), NC (2) NR	- + - -	NSAIDs ˉ IL-6, TNFα and PGE2, and IL-1β	NSAIDs effect on MMPs and TIMPs ?

Table 1: Production of biochemical factors by peri-prosthetic tissue cells (*Continued*)

Article - first author - last author - year	**Patients** - number (m/f) - age (yrs)* - primary diagnosis (n) - time to revision (yrs)*	**Prosthesis** - type (n) - fixation (n) - material	**Study material** - IFT tissue/cells - CM - bone cells - immune cells	**Study outcome – Cytokine/Matrix production** Cytokines / Chemokines	 Matrix molecules / Enzymes
Qian Jiang 2007 [39]	6 (3/3) 72 OA 8 (3 – 13)	THA C NR	+ + - -	Substance P in Fibs IL-6 and PGE2	
Qian Jiang 2008 [38]	8 (6/2) 72 ± 11 OA 9 ± 5	THA C NR	+ + - -	Substance P in Fibs IL-1β and TNFα	
Vallés Vilaboa 2012 [55]	11 (1 – 10) 70 ± 12 OA (9), other (2) 11 ± 3	THA NC CoCrMo/PE	+ + + +	IL-6 during culturing Hsp72 ˉ during culturing	
Niarakis Aletras 2013 [27]	NR NR NR NR	NR NR NR	+ - - -		MT1-MMP in Fibs by IL-1β or TNFα
Qian Wang 2013 [40]	8 (6/2) 72 ± 11 OA 9 ± 5	THA C NR	+ - - -	Substance P in Fibs RANKL	

Abbreviations: NR = not reported; IFT = interface tissue; CM = conditioned medium; OA = osteoarthritis; RA = rheumatoid arthritis; THA = total hip arthroplasty; TKA = total knee arthroplasty; C = cemented; NC = cementless; Fib = fibroblast; MQ = macrophage; Ti = titanium; PE = polyethylene; CoCr = cobalt chrome; Mo = Molybdenum; SS = stainless steel; + = yes/present; - = no/absent; ˉ/ = decrease/increase; </=/> = less/similar/more

* Data regarding age and time to revision are presented either as mean, mean (range), range, or mean ± SD, depending on the way of presentation in the original article

Collagenase and gelatinase were the most common matrix molecules/enzymes measured. An imbalance between matrix metalloproteinases (MMPs) and its tissue inhibitors (TIMPs) was reported to correlate with clinical severity of loosening.[35] In one study, matrix enzyme production was compared between hip and knee samples and tissue from failed knee prostheses was shown to produce higher enzyme levels than tissue from failed hip prostheses.[39] In addition, comparison of samples from cementless prostheses with samples of cemented ones revealed tissues from cementless knee prostheses to have the highest biochemical activity. However, in another study in which only tissues from hip prostheses were included, the cemented samples were observed to produce the highest enzyme levels.[35] The same study also reported on a tendency towards a higher gelatinolytic activity in tissue from the proximal region of the stem when compared to tissue from the distal part of the stem.

Response to particles

Response to wear particles has been addressed in numerous studies in literature, however, most of them use cell lines instead of primary cells. Consequently, our search strategy identified only 7 papers which reported on the response to particles using the target cells themselves (see Table 2). Fibrous tissue fibroblasts were used in 5 papers [17, 20, 21, 32, 43] and fibrous tissue macrophages were used in 2 papers.[23, 44] Response to particles, mostly Ti-particles, was studied by measuring the production of inflammatory mediators in 4 papers [21, 23, 32, 44], the production of matrix molecules in 1 paper [20], and the production of osteoclastogenic factors in 4 papers.[17, 21, 32, 43] Similar to macrophages, fibroblasts were shown to be able to respond to particles directly, possibly via phagocytosis.[32] Responsiveness was higher in fibrous tissue fibroblasts (and rheumatoid arthritis synovial fibroblasts) than in normal synovial fibroblasts.[21] Activation of fibroblasts was more extensive in cultures where conditioned medium from fibrous tissue membranes was added than in cultures where particles were added.[21] Since conditioned medium strongly enhanced the fibroblast response to particles [21, 32], inflammatory mediators in addition to particles seem essential for fibroblast activation.

Table 2: Response to particles by peri-prosthetic tissue cells *(Continued)*

Article - first author - last author - year	**Patients** - number (m/f) - age (yrs)* - primary diagnosis (n) - time to revision (yrs)*	**Prosthesis** - type (n) - fixation (n) - material	**Study material** - IFT tissue/cells - CM - bone cells - immune cells	**Study outcome – Response to particles** Cytokines / Chemokines	 Matrix molecules / Enzymes
Yao Galante 1995 [57]	6 (5/1) 36 – 74 OA (4), other (2) 3 – 12	THA NR CoCr, Ti	+ + + -		Ti mRNA of collagenase, stromelysin and MMP Response of IFT Fibs = fetal synovial Fibs CM of Ti-particle stimulated Fibs ¯ procollagen I and III mRNA in MG-63 cells
Al-Saffar Revell 1996 [2]	15 (6/9) 59 (34 – 85) OA (8), RA (4), other (3) 7 (2 – 15)	THA (10), TKA (5) C (6). NC (9) NR	+ + - -	Ti, but not PE, IL1β and GM-CSF mRNA by IFT MQs and Fibs	
Trindade Smith 2001 [53]	5 (NR) NR NR NR	NR NR NR	+ - - +	Ti, PMMA, and CoCr IL-6 and TNFα release by MQs but not lymphocytes dose of Ti and PMMA = cytokine levels dose of CoCr = unchanged cytokine levels	
Koreny Glant 2006 [21]	32 (18/14) 62 (34 – 91) OA (NR), RA (NR) 10	THA (23), TKA (9) NR NR	+ + - +	IFT Fibs phagocytose Ti particles Ti IL-1β, IL-6, IL-8, TNFα, MCP-1 and VEGF levels Ti + CM cytokine levels, but also of mCSF, RANKL and OPG	

Table 2: Response to particles by peri-prosthetic tissue cells *(Continued)*

Article - first author - last author - year	**Patients** - number (m/f) - age (yrs)* - primary diagnosis (n) - time to revision (yrs)*	**Prosthesis** - type (n) - fixation (n) - material	**Study material** - IFT tissue/cells - CM - bone cells - immune cells	**Study outcome – Response to particles** Cytokines / Chemokines	 Matrix molecules / Enzymes
Sun Fan 2006 [50]	7 (4/3) 72 NR NR	THA NR NR	+ + - +	Bone-cement IL-6, TNFα and RANKL secretion, but not OPG ¯ RANKL secretion after anti-IL-6 plus anti-TNFα, but not individual antibodies	
Tunyogi-Csapo Glant 2007 [54]	32 (18/14) 62 (34 – 91) OA (NR), RA (NR) 10	THA (23), TKA (9) NR NR	+ + - -	Ti mRNA of inflammatory, angiogenic and osteoclastogenic factors by IFT Fibs Response of IFT Fibs > normal synovial Fibs	
Qian Wang 2013 [40]	8 (6/2) 72 ± 11 OA 9 ± 5	THA C NR	+ - - -	Ti RANKL secretion by IFT Fibs Ti + SP RANKL secretion by IFT Fibs RANKL secretion ¯ after Cox inhibitor	

Abbreviations: NR = not reported; IFT = interface tissue; CM = conditioned medium; OA = osteoarthritis; RA = rheumatoid arthritis; THA = total hip arthroplasty; TKA = total knee arthroplasty; C = cemented; NC = cementless; Fib = fibroblast; MQ = macrophage; Ti = titanium; PE = polyethylene; PMMA = polymethylmethacrylate; CoCr = cobalt chrome; + = yes/present; - = no/absent; ¯/ = decrease/increase; </=/> = less/similar/more

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Osteoclastogenic capacity

Table 3 summarizes the findings of the 18 papers focusing on the osteoclastogenic capacity of cells from the peri-prosthetic fibrous tissue. Osteoclastogenic capacity was measured indirectly in 6 papers [25, 27, 29, 45-47] and directly in 12 papers.[15, 32, 35, 43, 48-55] Indirect investigation of the osteoclastogenic capacity was performed by studying the effect of conditioned medium on the release of radioactive calcium from bone specimens (^{45}Ca release). Conditioned medium obtained from capsular tissue cultures was shown to produce higher amounts of factors stimulating ^{45}Ca release than that obtained from membrane cultures.[47] In addition, conditioned medium from deeper layers of the capsule increased ^{45}Ca release more than that from superficial layers of the capsule.[27] Generally, only a few samples of conditioned media obtained from membranes affected ^{45}Ca release, which was observed not to differ between samples from failed hip and knee prostheses.[29]

Direct investigation of the osteoclastogenic capacity was performed by studying the effect of cells from the fibrous tissue on ^{45}Ca release in 2 papers [35, 52] and by studying their capability to induce resorption pits in 9 papers.[15, 43, 48-51, 53-55] Data regarding the effect of cells from fibrous membranes on ^{45}Ca release were conflicting, with one study showing an induction [52] and another showing no effect.[35] No papers reported on ^{45}Ca release by capsule cells. Induction of the formation of resorption pits was reported for both capsule cells [43, 48, 49, 51] and membrane cells.[15, 49, 50, 54, 55]

In one study only the formation of $TRAP^{+}$ multinucleated cells (osteoclasts) was determined and no functional tests were performed.[32] In this paper, as well as in most of the other papers mentioned above, cells from the fibrous tissue were reported to become osteoclasts after stimulation only. Formation of osteoclasts from fibrous tissue macrophages was reported to be RANKL dependent when cultured in the absence of mCSF [15, 49, 50] and TNFα dependent when cultured in the presence of mCSF.[54] Fibrous tissue fibroblasts were shown to support osteoclast formation of precursor cells rather than to be able to become osteoclasts themselves.[43, 51, 55] Generally this supporting activity was reported to be dependent upon cell-cell contact, however, one study showed osteoclast formation to occur without direct cell-cell contact.[43] Only one study showed fibrous tissue fibroblasts themselves to be capable to actively resorb bone.[53]

Table 3: Osteoclastogenic capacity of peri-prosthetic tissue cells

Article - first author - last author - year	**Patients** - number (m/f) - age (yrs)* - primary diagnosis (n) - time to revision (yrs)*	**Prosthesis** - type (n) - fixation (n) - material	**Study material** - IFT tissue/cells - CM - bone cells - immune cells	**Study outcome – Osteoclastogenesis**
Goldring Harris 1986 [11]	41 (NR) NR OA NR	THA C NR	+ + + -	^{45}Ca and resorbing activity by membrane CM, which did not correlate with PGE2 levels Indomethacin $^{-}$ ^{45}Ca
Appel Herman 1990 [3]	30 (16/14) 60 (22 – 83) OA 7 (2 – 15)	THA C NR	- + - -	^{45}Ca by 12 of 16 membrane CMs
Ohlin Lerner 1990 [29]	6 (NR) 68 OA (4), other (2) 10	THA C NR	- + - -	^{45}Ca by capsule CM, but only when CM was isolated early during culture ^{45}Ca by 2 of 3 membrane CMs, 1 membrane CM $^{-}$ ^{45}Ca
Athanasou Bulstrode 1992 [4]	4 (NR) NR NR NR	THA NR NR	+ - - -	Capsule cells were TRAP^{+} and anti-CD68^{+} mononuclear cells, which became multinucleated after contact with bone Cells were capable to form few resorption pits
Ohlin Lerner 1993 [30]	13 (7/6) 70 OA 9	THA NR CoCrMo, Ti	- + - -	^{45}Ca by 12 of 13 capsule CMs and by 6 of 6 proximal femur membrane CMs, but only by 6 of 11 acetabular membrane CMs ^{45}Ca $^{-}$ by indomethacin, flurbiprofen, meclophenamic acid, dexamethasone, hydrocortisone and calcitonin
Ohlin Lerner 1994 [31]	5 (1/4) 67 OA (4), other (1) 13	THA NR Delrin$^{\circ}$, PE	+ + - -	^{45}Ca and PGE2 were higher in intermediate and deep capsule layers than in the superficial part of the capsule ^{45}Ca $^{-}$ by indomethacin
Yokohama Okada 1995 [58]	34 (2/32) 72 (41 – 74) NR 8 (3 – 20)	THA C (15), NC (19) CoCr, Ti	+ + - -	^{45}Ca in IFT cells = mouse Fibs , but < mouse MQs ^{45}Ca in cemented IFTs = non-cemented IFTs

Table 3: Osteoclastogenic capacity of peri-prosthetic tissue cells *(Continued)*

Article - first author - last author - year	**Patients** - number (m/f) - age (yrs)* - primary diagnosis (n) - time to revision (yrs)*	**Prosthesis** - type (n) - fixation (n) - material	**Study material** - IFT tissue/cells - CM - bone cells - immune cells	**Study outcome – Osteoclastogenesis**
Perry Elson 1996 [34]	40 (13/27) 74 (38 – 89) OA (32), RA (2), other (6) 11 (1 – 22)	THA (29), TKA (11) C (38), NC (2) NR	+ + - -	^{45}Ca by 23 of 40 IFT CMs, $^{-}$ by 1, and not affected by 16 Addition of anti-IL1 to CMs did not change ^{45}Ca release Hips = knees
Sabokbar Athanasou 1997 [41]	7 (5/2) 68 NR 8	THA NR CoCr, Ti, SS, PE, PMMA, Ceramic	+ - + -	IFT cells were CD11b^{+}, CD14^{+}, TRAP^{+} and VNR^{+} after co-culturing with rat bone cells and VitD, and were capable of extensive pit formation Anti-mCSF $^{-}$ TRAP, VNR and pit formation
Neale Athanasou 1999 [26]	9 (3/6) 74 NR 10	THA C (8), NC (1) CoCr, Ti, SS, PE	+ + + -	Anti-IL-1β nor anti-TNFα inhibits TRAP, VNR and pit formation. Anti-mCSF and anti-IL6 do, but not additively Inhibition acts only during early stages of osteoclast differentiation
Itonaga Athanasou 2000 [18]	6 (3/3) 48 – 85 OA NR	THA NR NR	+ + + +	Pseudomembrane cells were TRAP^{+}, VNR^{+} pit forming cells in presence of RANKL, no mCSF or bone cells were needed mCSF plus dexamethasone TRAP, VNR and pit formation OPG $^{-}$ TRAP, VNR and pit formation
Sakai Iwamoto 2002 [45]	7 (3/4) 73 NR NR	THA NR NR	+ - + +	Granulation Fibs itself are not TRAP^{+}, but induce TRAP, CTR and pit formation by bone marrow cells
Ong Taylor 2003 [32]	6 (1/5) 69 OA 12	THA NR CoCr, Ti, Ceramic, PMMA	+ - - -	^{45}Ca by IFT cells ^{45}Ca $^{-}$ by doxycycline

Table 3: Osteoclastogenic capacity of peri-prosthetic tissue cells *(Continued)*

Article - first author - last author - year	**Patients** - number (m/f) - age (yrs)* - primary diagnosis (n) - time to revision (yrs)*	**Prosthesis** - type (n) - fixation (n) - material	**Study material** - IFT tissue/cells - CM - bone cells - immune cells	**Study outcome – Osteoclastogenesis**
Pap Aicher 2003 [33]	5 (NR) NR NR NR	NR NR NR	+ - - +	IFT Fibs are capable of pit formation TNFα pit formation
Sabokbar Athanasou 2003 [43]	8 (4/4) 48 – 85 NR NR	THA NR NR	+ - - +	TRAP$^+$ and pit formation by IFT cells in presence of TNFα (+/- IL-1α) and even more in presence of RANKL OPG, RANK:Fc and TNFRp75 could not inhibit TRAP and pit formation, while TNFRp55 could
Sabokbar Athanasou 2005 [42]	10 (4/6) 48 – 85 OA 8	THA C (7), NC (3) NR	+ - - +	TRAP$^+$ and pit formation by IFT Fibs plus PBMCs plus mCSF, but not in transwell cultures OPG and anti-TNFα alone reduced TRAP and pit formation, while the combination completely abolished TRAP and pit formation
Koreny Glant 2006 [21]	32 (18/14) 62 (34 – 91) OA (NR), RA (NR) 10	THA (23), TKA (9) NR NR	+ + - +	TRAP$^+$ by IFT cells plus bone marrow-derived stromal cells plus mCSF, and by titanium-stimulated IFT Fibs plus bone marrow-derived stromal cells plus mCSF
Sun Fan 2006 [50]	7 (4/3) 72 NR NR	THA NR NR	+ + - +	TRAP$^+$ and pit formation in co-cultures of pseudocapsular Fibs plus PBMCs, also in transwell cultures but to a lesser extent Bone-cement particles pit formation Anti-IL6 and anti-TNFα alone did not affect pit formation, while the combination partially blocked pit formation. RANK:Fc even completely blocked pit formation

Abbreviations: NR = not reported; IFT = interface tissue; CM = conditioned medium; OA = osteoarthritis; RA = rheumatoid arthritis; THA = total hip arthroplasty; TKA = total knee arthroplasty; C = cemented; NC = cementless; TRAP = Tartrate-resistant acid phosphatase; CTR = Calcitonin receptor; VNR = Vitronectin receptor; Fib = fibroblast; MQ = macrophage; PMBC = peripheral blood mononuclear cell; Ti = titanium; PE = polyethylene; CoCr = cobalt chrome; Mo = Molybdenum; SS = stainless steel; PMMA = polymethylmethacrylate; + = yes/present; - = no/absent; ˉ/ = decrease/increase; </=/> = less/similar/more

* Data regarding age and time to revision are presented either as mean, mean (range), range, or mean ± SD, depending on the way of presentation in the original article

Osteogenic capacity

Our search strategy revealed no papers in which the osteogenic capacity of peri-prosthetic fibrous tissue cells was explored *in vitro*. Of the included full-text papers, only one paper did present some data on the osteogenic potential of the cells. Al-Saffar et al. showed by histological assessment that the fibrous tissue of some patients exhibits osteogenic characteristics as shown by the presence of intramembranous formation of osteoid.[44] Cells present at these sites within the membranes were found to be fibroblasts and macrophages.

Two papers presented findings on the effect of conditioned medium obtained from peri-prosthetic tissue on osteoblasts. Yao et al. observed conditioned medium from Ti particle-stimulated peri-prosthetic fibroblast cultures to suppress the gene expression of procollagens by MG-63 osteosarcoma cells [20]. Ito et al. showed conditioned medium from a peri-prosthetic tissue culture to have no effect on collagen I gene expression, but to increase IL-6 gene expression in normal human osteoblasts.[31] Although these findings do not shed any light on the osteogenic potential of peri-prosthetic tissue cells, it does imply that (effects on) osteoblasts play a role in the process of aseptic loosening.

Discussion

This systematic review provides an overview of the characteristics of peri-prosthetic fibrous tissue based on *in vitro* findings. Fifty-one papers were thoroughly studied in order to extract data regarding the production of biochemical factors, response to particles, osteoclastogenic capacity, and osteogenic capacity of the tissue. Macrophages and fibroblasts were shown to act in concert in aseptic loosening: both cell types seem to be actively involved in osteoclastogenesis and pathologic bone resorption through production of inflammatory cytokines, chemokines, matrix degrading enzymes, osteoclastogenic factors and angiogenic factors. Some studies showed direct correlations of these parameters with the radiographic appearance or severity of loosening.[30, 35, 39, 40] Wear particles, in particular Ti-particles, interfered with all these parameters. However, the effect of particles on responses of the tissue was most powerful in the presence of inflammatory mediators.[21, 32]

An important limitation of the literature, but inextricably linked to research on peri-prosthetic tissue, is the large heterogeneity with respect to the reported findings. Part of the variability is most likely dependent on the type of prosthesis (hip versus knee) and type of fixation (cemented versus cementless). However, we found inconsistent findings with respect to these variables, with both differences between groups [28, 35, 37, 39] and comparable findings between groups [28, 29, 35, 38, 39, 41] reported. Part of the variability might also be explained by the cellular composition of peri-prosthetic fibrous tissue. However, this literature study showed that macrophages and fibroblasts, the main cell types of the tissue, both produce inflammatory mediators and matrix degrading molecules known to be important in the loosening process.[14-22] In addition, both cell types were shown to have the potential to induce osteoclastogenesis and bone resorption, either direct or indirect.[15, 32, 43, 49-55] Therefore, other patient- and tissue-related characteristics, generally not specified in papers such as type of bone loss (linear versus erosive [30]) and *in situ* location and orientation of the tissue (proximal versus distal [35], and superficial versus deep tissue layers [27]), may have contributed to the observed variation as well.

Another limitation of the literature is the large variation in measured study outcomes between different studies. Since '*in vitro* findings' is a broad term, some variation was already expected before performing the literature search. Therefore, *a priori* the study outcomes were divided into four categories: production of biochemical factors, response to particles, osteoclastogenic capacity, and osteogenic capacity. However, after data extraction, even within these categories a high diversity in measured variables was observed which caused difficulties in making comparisons between studies. Therefore, studies with a sufficient sample size and more detailed and comparable description of study characteristics should be conducted in order to greatly improve interpretation of results.

A limitation of the current study is the difficulty to develop a comprehensive search strategy. The denomination of peri-prosthetic fibrous tissue is far from uniform and the same holds true for the term '*in vitro* studies'. Therefore, potentially eligible studies may have been missed.

Nonetheless, fitting the pieces together leaves us with the reconstruction of a quite complex cellular mechanism involved in aseptic loosening (see Figure 2). From this proposed mechanism it becomes clear that only limited attention has been paid to the involvement of osteoblasts. Most research has focused on studying the interaction between particles and *in vitro* cell line models, which is beyond the scope of this review as we were interested in responses of primary peri-prosthetic tissue cells. The few papers studying cells obtained from peri-prosthetic fibrous tissue, showed these cells to produce factors that suppress osteoblast function and to induce production of inflammatory cytokines. [20, 31] In addition, peri-prosthetic tissue cells were shown to possibly exhibit osteogenic characteristics themselves.[44] These results triggered us to perform an additional PubMed search specifically on osteogenesis in aseptic loosening to be sure we did not miss any relevant literature on this topic. The search revealed another three relevant papers. The first [56] was an extension of previous findings already mentioned in the Results section.[44] In the second paper, molecular profiles of cells residing in the peri-prosthetic tissues were determined, which showed foamy macrophages to produce amongst others osteopontin, osteocalcin, osteonectin, alkaline phosphatase and type I collagen.[57] In contrast, spindle-shaped mesenchymal cells, possibly fibroblasts, failed to express these genes. These findings suggest an overlap in functions between peri-prosthetic tissue macrophages and osteoblasts or indicate an ability of these macrophages to transdifferentiate.

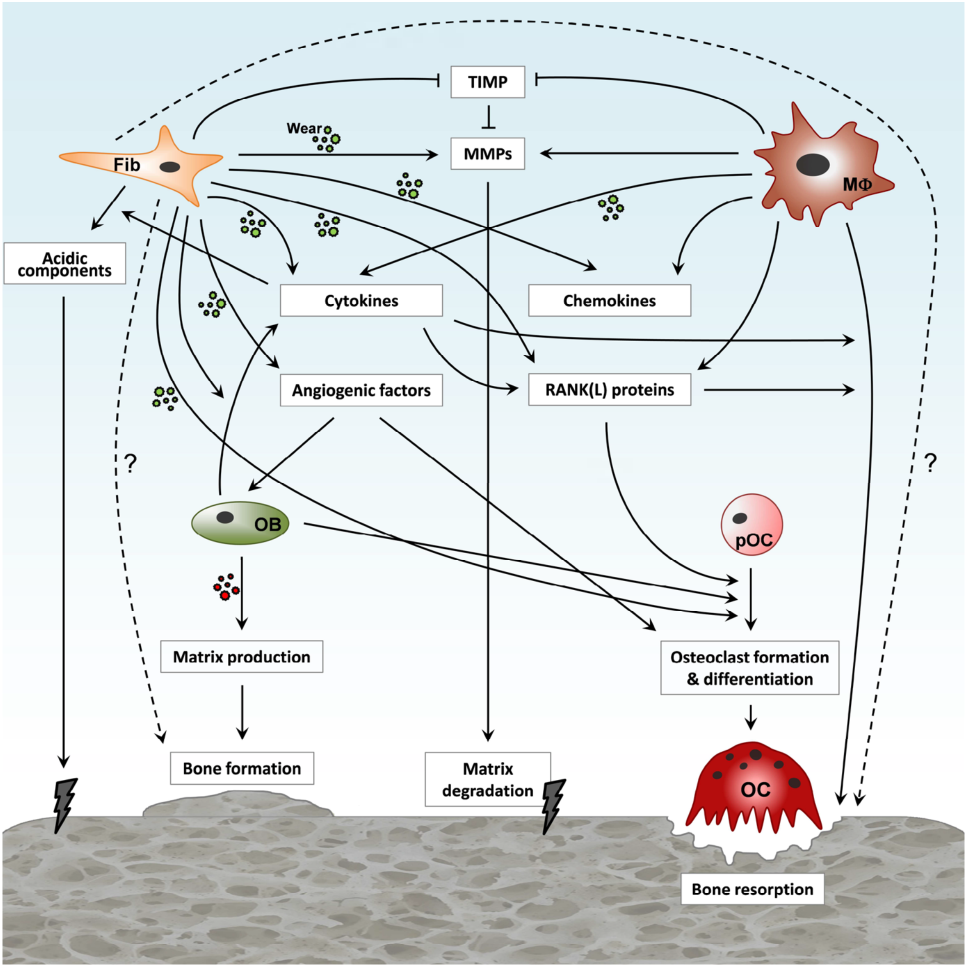

Figure 2: Complex cellular mechanisms involved in aseptic loosening. Fibroblasts and macrophages are the main cells involved in the process of aseptic loosening. Both cell types act in concert in inducing osteoclastogenesis and bone resorption through production of inflammatory cytokines, chemokines, matrix degrading enzymes, osteoclastogenic factors and angiogenic factors. In addition, fibroblasts seem to stimulate osteoblast function and bone formation through direct and indirect actions. Wear particles, in particular Ti-particles, interfere with many of these processes. The proposed mechanism depicted here, is solely based on the in this review included papers on *in vitro* findings using peri-prosthetic tissue cells. Additional (cellular) interactions and influences of wear have been described in the literature and might play a role in the process of osteolysis as well. However, those findings are either based on *in vitro* experiments using cell line models or are obtained by using other techniques than cell culture, and are as such beyond the scope of this review. An arrow indicates stimulation and a bar-headed line indicates inhibition. Green-colored particles indicate a stimulation of the respective process, whereas red-colored particles indicate an inhibition. The lightning sign depicts matrix degradation. The question mark represents findings reported by only one study.

Lastly, the third paper showed by expression analysis that two key regulators of osteogenesis, BMP-4 and FGF-18, were present at lower levels in peri-prosthetic fibrous tissue than in synovial tissue.[58] These findings suggest a disturbed osteogenic signalling in patients with osteolysis. Taken together, these data show that more research should be conducted to delineate the potentially critical role of osteoblasts in peri-prosthetic osteolysis.

Targets for treatment

Studies aiming at identifying targets for treatment of aseptic loosening have focused on blocking bone resorption, either directly or indirectly by inhibition of inflammation. The ability of blocking agents to inhibit bone resorption was observed to differ between studies and seemed to be dependent on the origin of cells (capsule vs. membrane), cell type (fibroblast vs. macrophage), and timing of addition of the blocking agent (early in differentiation vs. later on). Non-steroidal anti-inflammatory drugs (NSAIDs) such as tenidap and indomethacin were reported to inhibit IL-6, TNFα and PGE_2 production as well as to tend to inhibit MMP synthesis and stimulate TIMP production.[24, 42, 45, 47, 52] Although the effect of NSAIDs on all these mediators is favourable to reduce the resorptive process, bone resorption in aseptic loosening is too complex to be resolved by NSAIDs alone.[42, 47] Addition of antibodies to specific inflammatory mediators, such as TNFα and IL-6 antagonists, also resulted at the most in partial inhibition of bone resorption.[15, 43, 54, 55] Despite an important role for the RANK/RANKL signalling in aseptic loosening has been widely accepted, OPG, a decoy receptor for RANKL, alone was shown not to be able to fully block bone resorption.[50] One paper reported RANK:Fc, a RANKL antagonist, alone to be sufficient to block bone resorption completely [43], whereas others showed a combination of RANK:Fc and a TNFα antagonist is required to completely abolish bone resorption.[54, 55] Taken together, combination therapy seems inevitable to treat bone resorption in aseptic loosening.

Peri-prosthetic tissue fibroblasts were reported to play an important role in the process of aseptic loosening. These cells were shown to produce a wide range of inflammatory mediators, as well as OPG and RANKL, whereby supporting osteoclast formation and differentiation.[16-19, 21] In addition, peri-prosthetic tissue fibroblasts were observed to release MMPs and acidic components, as such contributing to the degradation of bone matrix.[20, 22, 35, 53] Therefore, fibroblasts should be considered a potential target for prevention of bone resorption in prosthesis loosening.

An animal model showed disturbances in biosynthetic processes to be at least as important in implant loosening as biodegradative processes.[9] Despite too little attention is being paid to the role of osteoblasts and bone formation in aseptic loosening, as shown in this review, the few studies that did address this topic showed a potentially critical role for this cell. A recent review on the role of osteoblasts in peri-prosthetic osteolysis, reported wear debris to significantly affect both (pre)osteoblast proliferation and function.[59] Moreover, particles are known to induce osteoblasts to secrete a variety of inflammatory mediators which contribute to the inflammatory cascade that drives osteolysis. Therefore, osteoblasts seem a potential therapeutic target in treating aseptic loosening as well.

Concluding remarks

The cellular mechanism of aseptic loosening is complex and involves the cross-talk between a variety of cells and the subsequent production of a wide-range of inflammatory mediators and matrix degrading factors. Besides the well-known role of macrophages and osteoclasts in the loosening process, the role of fibroblasts and osteoblasts should not be underestimated. High variability in all reported outcome measures was generally observed, which frequently hampered interpretation of the results. Therefore, future studies should aim to provide a more uniform and in-depth description of prosthesis-, patient-, and tissue-related characteristics in order to address the mechanism of aseptic loosening and its potential therapeutic targets more effectively.

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Supplementary data S-1 – Search strategy (April 7th, 2014)

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CELLULAR CONTENT OF PERI-PROSTHETIC TISSUE USING RT-PCR AND (IMMUNO)HISTOCHEMISTRY

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Submitted.

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Abstract

Numerous studies have investigated the cellular content and/or inflammatory mediators within peri-prosthetic tissue to explore the biological mechanisms underlying the aseptic loosening process. However, these studies generally have a small sample size and/or focus on specific cells or cell products within the tissue. Therefore, in this study, 47 peri-prosthetic tissue samples were analysed using RT-PCR as well as (immuno)histochemistry with a broad panel of cell specific genes and antibodies. Principal component analysis (PCA) was applied to the gene expression data to reduce the correlated data of individual genes and identified 2 components clustering fibroblast and osteoblast related genes in another component than macrophage and endothelial cell related genes. Overall, a high inter-tissue sample variability in factor loading scores of the components was observed, which could not be explained by patient- or prosthesis characteristics. (Immuno)histochemical staining of the tissue samples showed the predominant presence of both fibroblasts and macrophages with high inter- and intra-tissue sample variation in stained area and staining location. No significant associations were found between the stained area and patient- or prosthesis characteristics or gene expression data. Besides macrophages in general, the presence of macrophage-subtypes were also studied. In one-third of the samples M1 and M2 macrophages were present in comparable amounts, whereas almost two-third showed the predominance of M2 macrophages. In conclusion, fibroblasts and osteoblasts seems to be at least as important as macrophages in the aseptic loosening process. In addition, in particular M2 macrophages seemed to be present in the peri-prosthetic tissue.

Keywords: peri-prosthetic tissue, RT-PCR, (immuno)histochemisty, macrophage-subtypes, fibroblasts.

Introduction

Aseptic loosening is the main long-term adverse event associated with total joint arthroplasties.[1] Although several theories on the process of loosening have been proposed, the contribution of wear-debris particles has been implicated to be the most important factor.[2] Particulate wear debris can be phagocytized by several cell types, including macrophages, fibroblasts, lymphocytes and osteoclasts, triggering a continuous inflammatory-like response with the production of pro-inflammatory and osteoclastogenic cytokines.[3] These mediators create a microenvironment that favours osteoclastogenesis and subsequently bone resorption, which can ultimately lead to prosthesis loosening. Often during the loosening process a fibrous-like peri-prosthetic tissue layer with poor mechanical properties is formed. Although many reports have provided information about the cellular content and/or inflammatory mediators within this tissue [4-6], the precise biological mechanisms underlying the loosening process have still not been elucidated completely. Thorough analysis of the peri-prosthetic tissue is therefore still warranted to better understand the biological responses around aseptic loosened prostheses.

Analysis of peri-prosthetic tissue has predominantly been performed using (immuno) histochemistry. Based on tissue appearance and cellular content, Morawietz et al. even proposed a standardized histopathological classification system for evaluation of peri-prosthetic tissue, which discriminates four different tissue subtypes.[7] The classification system is particularly useful to discriminate between septic and aseptic loosening. Discrimination between septic and aseptic loosening has also been studied using gene expression analysis, where several differentially expressed genes were found.[8] Gene expression analysis has also proven useful in studying the pathogenesis of aseptic loosening specifically. Only a few of these studies addressed the cell content of the tissue by gene expression analysis. However, these studies generally focused either on the expression patterns of inflammatory and osteoclastogenic molecules (e.g. chemokine ligand 18 (CCL18), tumor necrosis factor alpha (TNFα), matrix metalloproteinases (MMPs) and tissue inhibitors of metalloproteinase (TIMPs)) or on the expression of regulators of bone homeostasis (e.g. receptor activator of nuclear factor kappa-B ligand (RANKL), osteoprotegerin (OPG), bone morphogenetic protein 4 (BMP4) and fibroblast growth factor 18 (FGF18)).[9-13] In addition, these studies generally have a small sample size and/or focus on specific cells or cell products within the tissue (e.g. macrophages, giant cells and fibroblast-like cells).

To obtain new or additional insights in the pathogenesis of aseptic loosening, we explored the cellular content of peri-prosthetic tissue in a relatively large sample size. We used qRT-PCR to identify cell-specific gene expression patterns in peri-prosthetic tissues next to the more commonly used technique of (immuno)histochemistry using a broad panel of cell specific genes or antibodies.

Materials and methods

Peri-prosthetic tissue samples

Peri-prosthetic tissue samples harvested from aseptically loosened femoral stems or acetabular components of 47 patients were obtained during revision surgery of total hip replacements. The peri-prosthetic tissue was collected as "waste" material and as such should not be traceable to specific patients. Therefore, due to Dutch Medical Ethics laws and legislation, only limited donor characteristics are available (see Table 1). Collected samples were kept in sterile NaCl 0.9% at 4°C, for a maximum of 24 hours, until they were processed in two ways. One portion was immediately submerged in RNAlater® (RNA stabilization Reagent; Qiagen Inc., Valencia, CA) to maintain RNA integrity and stored at -20°C. A second portion was fixed in 3.7% buffered formaldehyde and embedded in paraffin. Previous studies have noted morphological variability in cellularity and composition of samples taken from peri-prosthetic tissue.[5, 7] To account for this variability, both tissue portions consisted of material from several regions within each harvested sample. The study was approved by the Medical Ethics Committee of Leiden University Medical Center (C12-107).

Quantitative RT-PCR

Tissue samples (~0.2-0.4 gr) were snap frozen in liquid nitrogen and homogenized on ice using a pounder. Total RNA was isolated from cells using RNA-Bee (Tel-Test Inc., Friendswood, TX, USA). cDNA was synthesized using M-MLV reverse transcriptase (Promega, Fitchburg, WI, USA) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the Quantitect SYBRgreen PCR kit (Qiagen, Venlo, The Netherlands) with an iQ5 PCR cycler (BioRad, Hercules, CA, USA). Cell specific primer sets, all spanning at least one intron, were developed for osteoblasts, fibroblasts, endothelial cells and macrophages (see Table 2). Data were normalized relative to GAPDH expression. Levels of gene expression were expressed as fold-change relative to expression in cell type specific positive controls (e.g. SaOS-2 cells, HDFA cells, human endothelial cells and human monocytes respectively) using the $2^{-\Delta\Delta Cq}$ method. RNA samples of 15 out of 47 donors were not suitable for analysis as result of low RNA quality or quantity or absence of GAPDH expression (needed for normalization). Of the missing donors, RNA isolated from explant cultures (passage 0) was used. Again, not all samples had sufficient RNA quality or quantity or lacked GAPDH expression. In total, samples of 41 donors could be used in gene expression analyses.

Table 1: Demographic data from included peri-prosthetic tissue samples.

Donor	Sex	Age	Bearing	Fixation	Years in situ	Cup/Stem
1	F	81	Metal-PE	Cemented	>5	Cup
2	M	74	Metal-PE	Cementless	>5	Cup
3	F	85	Metal-PE	Cemented	>5	Cup
4	M	74	CE-CE	Cementless	>5	Stem
5	M	74	CE-PE	Cementless	>5	Cup
6	F	80	Metal-PE	Cemented	>5	Stem
7	M	81	N/A	Cemented	>5	Stem
8*	F	58	Metal-PE	Cementless	2-5	Cup
9	F	82	Metal-PE	Cemented	>5	Cup
10	F	82	Metal-Metal	Cemented	>5	Cup
11	M	55	N/A	N/A	N/A	N/A
12*	M	75	Metal-PE	Cementless	>5	Stem
13	F	75	Metal-PE	Cementless	>5	Cup
14	F	80	Metal-PE	Cementless	>5	Cup
15*	M	66	CE-PE	Cementless	>5	N/A
16*	M	84	Metal-PE	Cemented	>5	Cup
17	M	59	Metal-PE	Cemented	>5	Stem
18	M	81	Metal-PE	Cemented	>5	Cup
19*	M	81	Metal-PE	Cemented	>5	Stem
20*	F	92	Metal-PE	Cemented	>5	Stem
21	F	79	Metal-PE	Cemented	>5	Stem
22	M	55	Metal-Metal	Cementless	>5	Cup
23*	F	73	Metal-PE	Cemented	>5	Cup
24	F	80	Metal-PE	Cemented	>5	Cup
25	F	73	N/A	Cementless	>5	Stem
26*	F	75	CE-PE	Cementless	>5	Cup
27*	F	82	Metal-PE	Cemented	>5	Cup
28	M	79	Metal-PE	Cemented	>5	Stem+Cup
29	F	80	Metal-PE	Cementless	>5	Cup
30*	F	86	CE-PE	Cemented	>5	Cup
31	M	32	Metal-PE	Cemented	2-5	Cup
32	F	76	CE-PE	Cementless	>5	Cup
33*	F	74	Metal-PE	Cemented	>5	Stem+Cup
34	M	69	Metal-PE	Cemented	>5	N/A
35	M	84	Metal-PE	Cemented	>5	Stem
36	F	71	Metal-PE	Cementless	>5	N/A
37	M	66	Metal-PE	Cemented	>5	Cup
38	F	80	Metal-PE	Cemented	2-5	Stem
39	F	81	Metal-PE	Cemented	>5	Stem

Table 1: Demographic data from included peri-prosthetic tissue samples. *(Continued)*

Donor	Sex	Age	Bearing	Fixation	Years in situ	Cup/Stem
40	F	95	Metal-PE	Cemented	>5	Cup
41*	M	71	CE-PE	Cementless	>5	N/A
42*	F	81	CE-PE	Cemented	>5	Cup
43*	M	64	CE-PE	Cementless	>5	Stem+Cup
44*	F	76	Metal-PE	Cementless	>5	Cup
45*	F	63	Metal-PE	Cementless	>5	Cup
46	F	48	Metal-PE	Cemented	>5	Cup

F = Female, M = Male, PE = Polyethylene, CE = Ceramics, N/A = not available, *Could not be used in PCA analysis

Table 2: Oligonucleotides used in RT-PCR

Gene	Forward	Reverse
Glyceraldehyde 3-phosphate dehydrogenase (GAPDH)	5'-GACAGTCAGCCGCATCTTC-'3	5'-GCAACAATATCCACTTTACCAGAG-'3
Alkaline Phosphatase (ALP)	5'-TAAAGCAGGTCTTGGGGTGC-'3	5'-GGGTCTTTCTCTTTCTCTGGCA-'3
Osteocalcin (OCN)	5'-CCCAGCGGTGCAGAGTC-'3	5'-TCAGCCAACTCGTCACAGTC-'3
S100 calcium-binding protein A4 (S100A4)	5'-TTGGTTTGGTGCTTCTGAGATGT-'3	5'- TCACCCTCTTTGCCCGAGTA-'3
Vimentin (VIM)	5'- CCAAACTTTTCCTCCCTGAACC-'3	5'- CGTGATGCTGAGAAGTTTCGTTGA-'3
Endoglin (ENG = CD105)	5'- TCACCACAGCGGAAAAAGGT-'3	5'- CAGGAACTCGGAGACGGATG-'3
Platelet endothelial cell adhesion molecule (PECAM-1 = CD31)	5'- AGACGTGCAGTACACGGAAG-'3	5'- CTTTCCACGGCATCAGGGA-'3
Cluster of Differentiation 68 (CD68)	5'- AGGCTGGCTGTGCTTTTCTC-'3	5'- TCTCTGTAACCGTGGGTGTC-'3

Histology and (immuno)histochemistry

Serial tissue sections of 6 µm thickness were cut using a microtome (RM2235, Leica, Germany). Composition and cellularity were assessed using a haematoxylin and eosin (HE) stain. To determine the presence of a variety of different cell types, immunohistochemical staining was carried out using antibodies directed against CD68 (prediluted; Abcam, Cambridge, United Kingdom), a pan macrophage marker; CD86 (1:200; GeneTex, Irvine, CA, USA), a marker for M1 macrophages[14]; CD206 (1:500; Abcam), a marker for M2 macrophages[15]; CD31 (1:500; Dako, Hamburg, Germany), a marker for endothelial cells; vimentin (1:200; Dako), a marker for fibroblasts and SATB2 (1:300; Atlas Antibodies, Stockholm, Sweden), a marker for osteoblasts.[16] Furthermore, Alizarin Red S (20mg/mL; Sigma-Aldrich, St Louis, MO, USA) was used to stain calcium deposits (mineralization) in the tissue, as indirect marker for the presence of osteoblasts. Details on staining procedures can be found in supplementary S1.

In total, tissue sections of 46 out of 47 donors could be used, as the sections of one donor were lost during almost every staining. Tissue sections were analysed with an Olympus BX43 light microscope (Olympus, Tokyo, Japan). Samples, stained for CD31, CD68 and vimentin, were ranked independently by two blinded observers based on their relative amount of positive stained area in three sections per sample, according to slight modifications on a previous protocol.[17] Briefly, ranking was done for each marker separately and samples were given a number between 1 (highest ranking) and 46 (lowest ranking) based on the visually estimated total stained area, and the total number of samples, namely 46. This resulted in two ranks from each observer per staining, which showed a >80% similarity in ranking scores. After consensus on the discrepancies with a third observer, one single rank was used for further analysis. Actual ranking of vimentin was hard due to relatively small differences in amount of stained area. However, the tissue samples could be subdivided into 3 groups representing either relatively little, intermediate or intensive amount of stained area.

For investigation of the proportion of macrophage subtypes within the tissue, we analysed the presence of CD68, CD86 and CD206 positive cells in the same tissue regions within each sample. In total tissue sections of 37 out of 46 were analysed, as tissue regions of these samples had CD68+ staining.

Statistical analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics 21). For analysis of the gene expression data, principal component analysis (PCA) was done to reduce the multivariate gene expression data down to create summary variables (called principal components).[18] The goal of this method is to reduce the total number of variables analysed by explaining most of the variation in the data by one or a few new variables. Relative gene expression data of alkaline phosphatase, osteocalcin, vimentin, S100A4, endoglin, PECAM-1 and CD68 were entered in the PCA. Percentage of variance explained by the components and Eigen values > 1 were used to determine the number of principal components. For each variable in the extracted component(s), a factor loading was calculated which can be interpreted as correlation measure between the observed variable and the underlying unobservable component. For analysis, only factor loading scores of > 0.4 qualified for loading of a variable on a component.[19] Factor loading scores of each donor were then plotted in a graph and classified as 'high' when factor loading scores were above the 75^{th} percentile, 'intermediate' when they were between the 75^{th} and 25^{th} percentile, and as 'low' when they were below the 25^{th} percentile. Linear regression analysis was used to study the relation between the factor loading scores and patient or prosthesis characteristics.

To compare between the histological ranking scores, patient- and prosthesis characteristics and the gene expression data, the Kruskal Wallis test, χ^2, one-way ANOVA or Spearman rank were used. For all tests, a p-value of <0.05 was regarded as statistically significant.

Results

All tissue samples available for PCR analysis (n=41) showed expression of GAPDH, vimentin, endoglin and CD68, whereas expression of alkaline phosphatase (n=36), osteocalcin (n=34), S100A4 (n=38) and PECAM-1 (n=39) was observed in nearly all of the tissue samples. Generally, we observed a high inter-donor variability in all gene expression levels, as shown in Figure 1. Additionally, high correlations between the relative mRNA expression levels of all genes were found (Supplementary Table 1).

Principal component analysis (PCA) was used to reduce the correlated gene expression data. As PCA requires the use of samples with complete relative expression data sets, only data from 30 tissue samples could be used. As shown in Table 3, two components were extracted. The first component is determined by a combination of vimentin, alkaline phosphatase, osteocalcin, S100A4 and endoglin, and explains 49.4% of the total variation. The second component, explaining 18.9% of the total variation, is determined by a combination of endoglin, PECAM-1 and CD68.

Next, we identified donors who either scored 'high' (factor loading scores above the 75th percentile) or 'low' (factor loading scores below the 25th percentile) in the PCA components by plotting the factor loading scores of both components (supplementary Figure 1). Two donors (7 and 11) scored 'high' in both components and two donors (2 and 28) scored 'low' in both components. One donor (6) scored 'high' in the first component and 'low' in the second component, whereas four donors (14, 21, 29, 36) scored 'low' in the first component and 'high' in the second component. Overall, most donors (n= 21) scored intermediate for both components.

Subsequently, we investigated the relationship between the extracted components and (several) patient- and prosthesis characteristics. No significant associations were found between gender and age of the donors and the extracted PCA components (Component 1, gender: β=0.021, p=0.681 and age: β=0.003, p=0.812 ; Component 2, gender: β=-0.196, p=0.613 and age: β=-0.010, p=0.524). The tissue samples scoring 'low' in the first component where observed to be primarily of donors who received an cementless prosthesis (5 out of 7), whereas samples of patients who received a cemented prosthesis generally scored relatively high in the first component (5 out of 7). Nevertheless, no significant association was found between type of fixation and the PCA components (Component 1: β=0.320, p=0.259; Component 2: β=-0.060, p=0.873). Unfortunately, we were not able to perform correlation analysis for type of bearing and the PCA components, since most of the donors (22 out of 30) received a prosthesis with polyethylene-metal bearing.

The presence of fibroblasts (vimentin+), endothelial cells (CD31+) and macrophages (CD68+) was shown in almost all samples. The presence of osteoblasts could not be shown using Special AT-rich sequence-binding protein 2 (SATB2), a recently discovered osteoblast marker which plays a critical role in osteoblast lineage commitment,[16] as this staining did not work in our hands. However, in a few tissue samples calcium deposits (Alizarin Red S+ stained areas) were observed. In general, a high inter- and intra-sample variability in terms of amount of stained area and localization was seen for all stainings. Next, we examined the tissue samples by ordering them based on the factor loading scores of either Component 1 or Component 2. Figure 2 shows representative figures of the (immuno)histochemical stainings based on classification (high, intermediate and low) of the tissue samples according to the PCA analysis. However, no conclusions could be drawn analysing the tissue samples this way. Therefore, we ranked (CD31 and CD68) or subdivided (vimentin) the tissue samples based on the visually estimated total stained area.

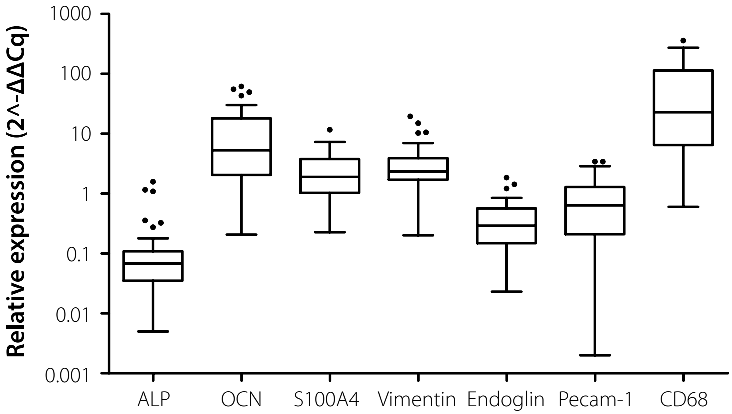

Figure 1: Relative gene expression levels (in Log 10 -scale) of cell related genes. Boxplots showing the relative expression of alkaline phosphatase (ALP; n=36), osteocalcin (OCN; n=34), S100A4(n=38), Vimentin (n=41), Endoglin (n=41), PECAM-1(n=39) and CD68 (n=41). Values represent mean ± SD, dots represents 1.5*IQR. IQR: Inter Quartile Range.

Table 3: Principal Component Analysis of gene expression data

Genes	Component[a]	
	1	2
ALP	0.833	
OCN	0.734	
S100A4	0.684	
Vimentin	0.838	
Endoglin	0.673	0.601
Pecam-1		0.864
CD68		0.812
Variance explained (%)	49.4	18.9

Components with 'Eigen values' >1 are extracted after Varimax rotation with Kaiser Normalization, significant coefficients with values >0.4 are displayed.

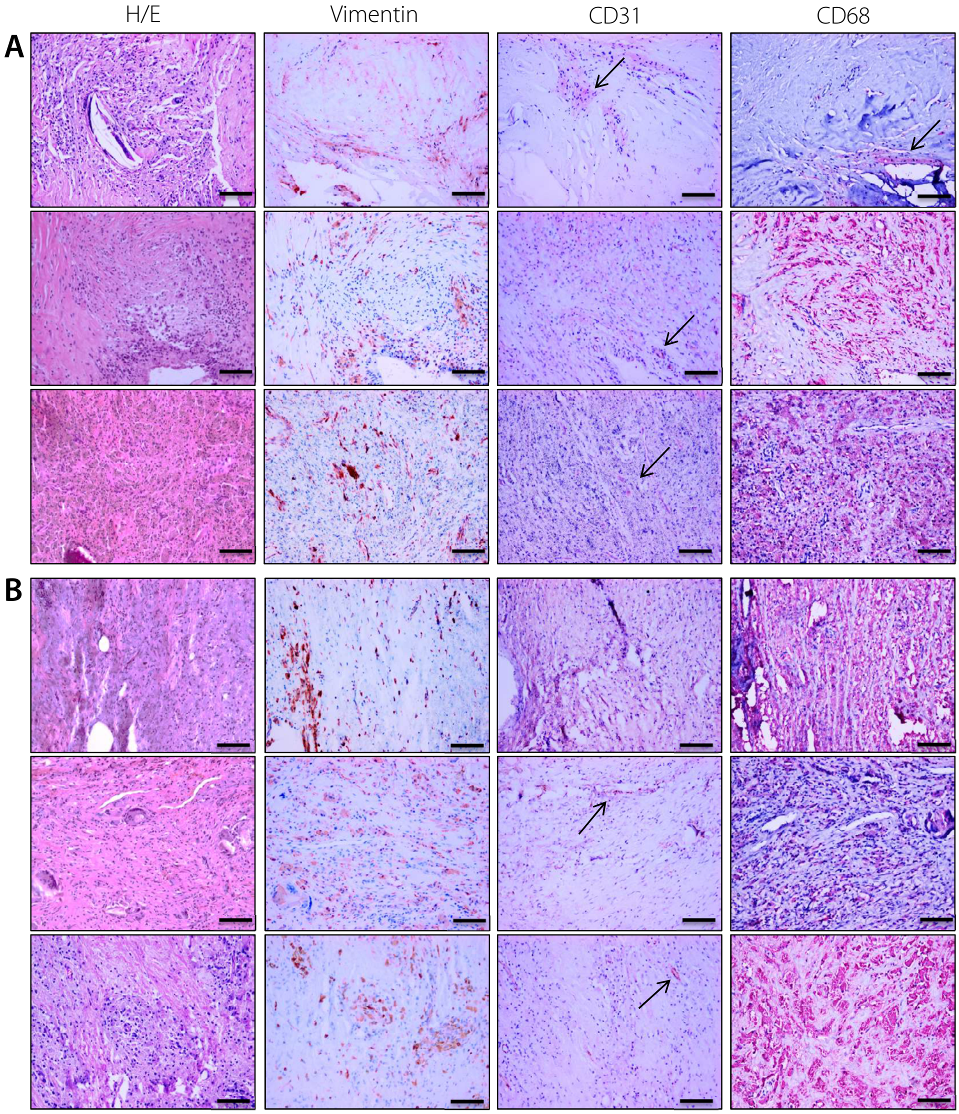

Figure 2: Representative images of (immuno)histochemical stainings based on factor loading scores according to the PCA analysis. Vimentin and CD31 positive cells are shown in red/brown. Cells positive for CD68 are red.

(A) Representative pictures of tissue samples which scored highest (top row, sample 11), intermediate (middle row, sample 40) and lowest (bottom row, sample 22) in Component 1 according to the PCA analysis.

(B) Representative pictures of tissue samples which scored highest (top row, sample 35), intermediate (middle row, sample 33) and lowest (bottom row, sample 6) in Component 2 according to the PCA analysis.

Bars represent 100µm. Arrows point to positively stained cells.

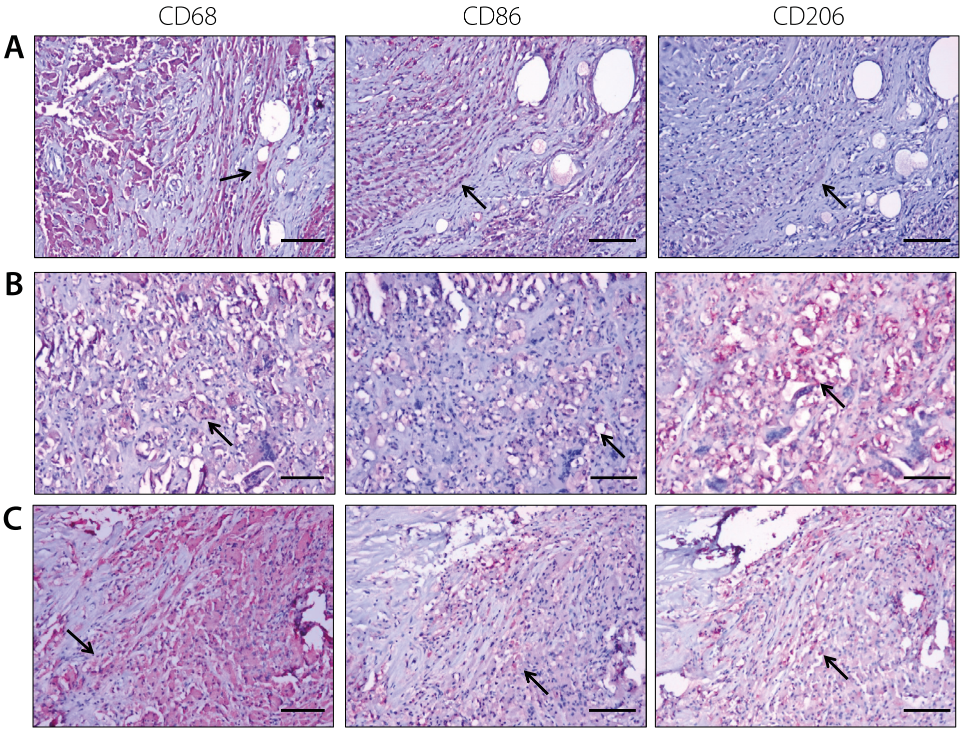

Figure 3: Immunohistochemical stainings of macrophage polarization. CD68, CD86 and CD206 positive cells are shown in red.
(A) Representative tissue sample (sample 10) with more M1 (CD86+) staining than M2 (CD206+) staining. (B) Representative tissue sample (sample 26) with more M2 staining than M1 staining. (C) Representative tissue sample (sample 41) with equal amount of M1 and M2 staining.
Bars represent 100μm. Arrows point to positively stained cells.

This allowed us to investigate the relation between the tissue sample stainings and patient- and prosthesis characteristics and the relation between gene expression data and histology data. CD31 and CD68 ranking was not significantly different between male and female nor between different types of fixation and did not correlate with age. Likewise, no significant associations were found between the subdivided vimentin staining and patient characteristics (gender or age) or type of fixation. Furthermore, no associations were found between the tissue sample stainings and the corresponding gene expression data or the factor loading scores of Component 1 and 2.

Next to the general macrophage marker CD68, we also stained the peri-prosthetic samples against CD86 and CD206, which are indicative for M1 and M2 macrophages, respectively. Both subsets were present within the tissue, however, large variation with respect to amount of stained area was observed. Representative figures are shown in Figure

3. In 37 tissue samples the relative proportion of CD86 and CD206 positive stained area/cells was determined. In 11 samples, no visual difference was observed between the amount of stained area of CD86 and CD206. Five samples showed more CD86 than CD206 stained area, whereas 21 samples showed more CD206 than CD86 stained area. No significant associations were found between the three groups (equal amount of CD86 and CD206 stained area, > CD86+ stained area or > CD206+ stained area) and gender and age. With respect to type of fixation, tissues from cemented prostheses were observed to be more likely to have more CD206+ stained area than tissues from cementless prostheses.

Discussion

In the current study, we explored the cellular content of peri-prosthetic tissue, obtained from aseptically loosened (hip)prostheses, using RT-PCR and (immuno)histochemistry. Since many cells are involved in the process of aseptic loosening, we studied a diverse panel of cell-specific markers. For RT-PCR, we assumed that a high RNA expression level of a certain gene was explained by the abundant presence of the cell type that usually expresses this gene. The variable presence of the studied genes between the tissue samples as well as the variation in levels of expression indicates a high variability in cellular composition between tissue samples. These results corroborate previous studies on the cellular content of peri-prosthetic tissues.[5, 7] We therefore took a further step in investigating the gene expression patterns between samples, using principal component analysis. This method revealed two components, clustering osteoblast- and fibroblast-related genes in the first component and macrophage- and endothelial cell-related genes in the second component. The clustering of osteoblast-related genes together with fibroblast-related genes in one component is not surprising since these cells are both from mesenchymal origin. In addition, fibroblasts have been reported to be able to express genes that are also expressed by osteoblasts.[20] Likewise, clustering of endothelial cell-related genes together with macrophage-related genes in the other component might be explained by the fact that inflammation is often accompanied by hypervascularization.[21, 22] The clustering of fibroblast and macrophage related genes in different components in our study population proposes the presence of two types of peri-prosthetic tissue: a fibrotic-like tissue predominantly containing fibroblasts and an inflammatory-like tissue containing macrophages as the major cellular component. Morawietz et al. already described the presence of distinct cell types in peri-prosthetic tissues resulting in different histological classifications which represent different mechanisms of prosthesis loosening.[7] Considering the fact that the first component explained almost 50% of the observed variation in gene expression in our study population, this might indicate that in end-stage loosening the presence of fibroblasts outweighs the presence of macrophages, emphasizing the important role of fibroblasts within the peri-prosthetic tissue and within the loosening process.

The observed variation in factor loading scores of the components between tissues showed differences in the proportion of cell populations within each tissue. Despite the identification of several samples with 'high' or 'low' factor loading scores of either component, the majority of the tissue samples had an 'intermediate' phenotype. Several studies have already shown that differences in the profiles of cell populations (and histological appearance) were dependent on prosthesis characteristics (e.g. fixation, type of bearing or prosthesis lifetime).[5-7, 23] In our study, patient- or prosthesis characteristics (i.e. age, gender, type of fixation) could not explain the observed variation in factor loading scores (representing different cellular profiles). Nevertheless, a 'high' factor loading score in Component 1 was more often observed in tissue samples from cemented implants, whereas a 'low' factor loading score of Component 1 was more often observed in tissues from cementless implants. This result is not in line with previous studies in which regions of fibroblastic connective tissue were often observed surrounding cementless implants, whereas in tissues obtained from cemented implants less fibroblasts and more macrophages were present.[5, 7, 23] Discrepancies between our study and previous studies can be caused by differences in methodologies, as well as sample size. Unfortunately, despite our relatively large number of samples, the sample size was still too small to perform proper association analyses between different clinical groups. Therefore, in future studies, larger sample sizes of peri-prosthetic tissue with more detailed patient- and implant- characteristics are essential to improve interpretation of results.

Immunohistochemical staining of the tissue samples showed the predominant presence of fibroblasts and macrophages, as already known from literature. However, also endothelial cells where observed in several tissue samples, which indicate the importance of vascularization in the process of loosening.[24] Unfortunately, we were unable to identify osteoblasts in the tissue using immunohistology. However, we have observed calcium deposits in the extracellular matrix of a few tissue samples using Alizarin Red S, which might indicate the presence of osteoblasts. Combined with our interesting findings at the gene expression level, this definitely warrants further study of the osteoblast at the protein level.

For each staining, a high inter- and intra-tissue sample variation regarding the amount of stained area as well as location of the staining was observed. As already discussed above with the gene expression data, these variations might be the result of differences in patient- and implant characteristics. However, no significant associations were found between the ranking scores of CD31, CD68 or the three vimentin groups and gender, age or prosthesis fixation.

The observed importance of CD68+ macrophages in the PCA analysis, along with the recent interest in macrophage polarization as potential therapeutic strategy for treatment of aseptic loosening, led us to study the subtypes of macrophages within the peri-prosthetic tissue. In our sample population, almost one-third of the samples showed no visual differences in amount of CD86+ and CD206+ stained area which indicates no differences in M1 and M2 macrophage presence. However, almost two-third was observed to mainly contain CD206+ areas, indicating a predominance of M2 macrophages within the tissue. Our immunohistochemistry results corroborate the results of a gene expression study by Koulouvaris et al[10] in which they concluded that in the end-stage of osteolysis, M2 macrophage activation predominates. However, other studies either showed a predominance of M1 macrophages within the peri-prosthetic tissue[25] or could not come to a conclusion regarding differences in presence of macrophage phenotypes.[26] Discrepancies between studies can be caused by differences in methodologies, patient- and prosthesis characteristics as well as sample size. Although more CD206 staining was observed in tissues obtained from cemented compared to cementless prostheses, no significant associations between macrophage subtype and study characteristics were observed. Therefore, the data concerning the macrophage phenotype in peri-implant tissues remain controversial and warrants further study.

In this study, we have analysed a diverse panel of cell specific markers, including markers for macrophage polarization, in peri-prosthetic tissue of loosened total hip implants. For this purpose we combined both gene expression analysis and immunohistochemistry, to strengthen this study. The heterogeneity in patient- and prosthesis characteristics in this study makes our study sample representative of the general population, however, it also hampers data analysis. Therefore, despite our relatively large sample size, associations were often not significant, probably due to a lack of statistical power. In addition, the lack of information on the exact *in situ* location and orientation of the tissue samples further complicated interpretation of the results, especially the histology results. Therefore, future studies should aim to provide a more in-depth description of prosthesis-, patient-, and tissue-related characteristics in order to address the mechanism of aseptic loosening more effectively.

In conclusion, in-depth investigation of the peri-prosthetic tissue using RT-PCR and (immuno)histochemistry showed fibroblasts and osteoblasts to be at least as important in the aseptic loosening process as macrophages. With respect to macrophages, in particular M2 macrophages seem to be involved in the end-stage loosening process. As high inter- and intra-variability was seen at all levels studied, further study is warranted for even more detailed delineation of the mechanisms in aseptic prosthesis loosening.

Acknowledgements

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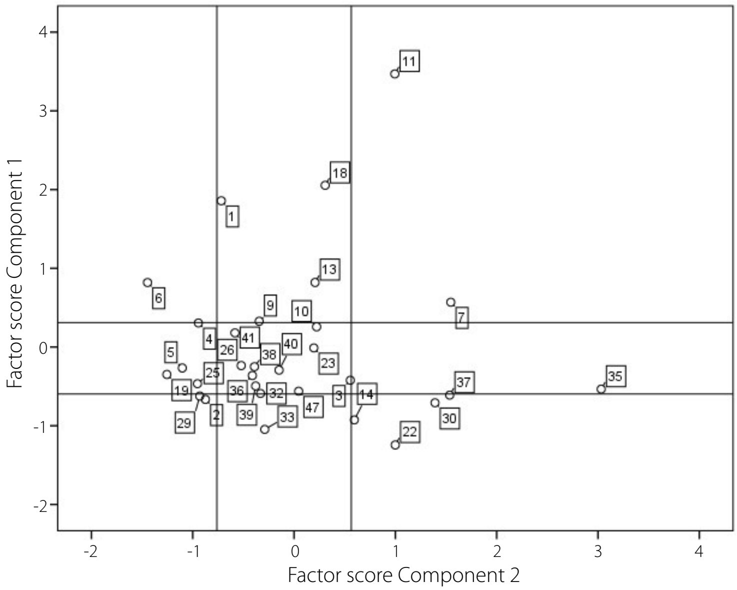
Supplementary data



Supplementary figure 1: Peri-prosthetic tissue sample plot of factor loading scores PCA analysis. Scatter plot of the factor scores extracted from the PCA analysis. Each dot represents one tissue sample. Lines represents 25th and 75th percentile of the component scores.

Supplementary table 1: Spearman correlations among gene expression data of peri-prosthetic tissue samples.

	P-Value						
	ALP	**OCN**	**S100A4**	**Vimentin**	**Endoglin**	**Pecam-1**	**CD68**
ALP		4.42×10^{-4}*	0.130	0.052	0.017*	0.044*	0.128
OCN	0.585		0.254	0.199	0.009*	0.025*	0.165
S100A4	0.261	0.204		0.001*	0.006*	0.054	0.446
Vimentin	0.326	0.226	0.534		2.11×10^{-4}*	0.002*	7.81×10^{-5}*
Endoglin	0.395	0.440	0.438	0.548		3.20×10^{-6}*	0.002*
Pecam-1	0.343	0.390	0.320	0.487	0.669		1.29×10^{-8}*
CD68	0.259	0.243	0.127	0.577	0.467	0.766	
	Spearman correlation						

*Significant at the p-values.

Supplementary methods

(Immuno)histochemical stainings of peri-prosthetic tissue

CD68, CD86 and CD206

After deparaffinization, antigen retrieval (CD68, CD206: citrate buffer pH6 ; CD86: citrate buffer pH9) was done followed by pre-incubation with 10% goat serum. Samples were then incubated (1hr) with monoclonal antibodies against CD68 (Abcam), CD86 (Genetex) and CD206 (Abcam), followed by incubation with link (30min) and label (30min) from the link-label kit (CD68: goat-anti mouse; CD86,CD206: goat-anti rabbit) (BioGenex, San Ramon, CA, USA). Freshly prepared neo-fuchsin was used as substrate.

CD31 and SATB2

After deparaffinization, tissue sections were blocked (30min) for endogenous peroxidase activity (0,3% Methanol/H_2O_2) followed by antigen retrieval (CD31: Proteinase-K 5µg/ml ; SATB2: citrate buffer pH6) and pre-incubation with 2,5% goat serum (CD31 only). Samples were then incubated (o/n) with monoclonal antibodies against CD31 and SATB2, followed by incubation with IMPRESS™ (CD31) (VECTOR) or EnVision™ (SATB2) (DAKO). Nova Red was used as a substrate. A cell was considered positive when stained red.

INHIBITION OF GSK3β STIMULATES BMP SIGNALLING AND DECREASES SOST EXPRESSION WHICH RESULTS IN ENHANCED OSTEOBLAST DIFFERENTIATION

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4

Abstract

Both bone morphogenetic protein (BMP) and Wnt signalling have significant roles in osteoblast differentiation and the interaction between BMP and Wnt signalling is well known. Sclerostin is an important inhibitor of bone formation, inhibiting Wnt signalling and downstream effects of BMP such as alkaline phosphatase activity and matrix mineralization in vitro. However, little is known about the effect of BMP and Wnt signalling interaction on the regulation of SOST, the gene encoding sclerostin. Possibly, uncoupling of osteoblast differentiation regulators and SOST expression could increase osteoblast differentiation. Therefore, we investigated the effect of BMP and Wnt signalling interaction on the expression of SOST and the subsequent effect on osteoblast differentiation. Human osteosarcoma cells (SaOS-2) and murine pre-osteoblast cells (KS483) were treated with different concentrations of Wnt3a, a specific GSK3β inhibitor (GIN) and BMP4. Both Wnt3a and GIN increased BMP4-induced BMP signalling and BMP4 increased Wnt3a and GIN-induced Wnt signalling. However, the effect of GIN was much stronger. Quantitative RT-PCR analysis showed that SOST expression dose-dependently decreased with increasing Wnt signalling, while BMP4 induced SOST expression. GIN significantly decreased the BMP4-induced SOST expression. This resulted in an increased osteoblast differentiation as measured by ALP activity in the medium and matrix mineralization. We conclude that GSK3β inhibition by GIN caused an uncoupling of BMP signalling and SOST expression, resulting in an increased BMP4-induced osteoblast differentiation. This effect can possibly be used in clinical practice to induce local bone formation, e.g. fracture healing or osseointegration of implants.

Keywords: osteoblast differentiation, SOST, sclerostin, BMP, GSK3β inhibition.

Introduction

Bone formation is a complex process that involves the differentiation of mesenchymal cells into pre-osteoblasts and osteoblasts that eventually leads to the synthesis and deposition of bone matrix proteins.[1] Bone is continuously remodelled by bone-forming osteoblasts and bone-resorbing osteoclasts.[2, 3] An imbalance in the remodelling process can result in bone diseases as sclerosteosis or osteoporosis. Osteoporosis is one of the most prevalent diseases in elderly [4] and is likely to become more prevalent with the further aging of the population. The expected higher prevalence of osteoporotic fractures and joint replacements due to the increase of the elderly population calls for the identification of regulatory molecules in differentiation of osteoblasts that can potentially serve as targets for treatment of osteoporosis. In addition, these molecules could possibly improve either fracture healing or osseointegration of implants.

Bone morphogenetic proteins (BMPs) and Wnts are well-known regulators of bone formation and have important roles in promoting osteoblast differentiation and mineralization. BMPs were identified as the factors responsible for induction of ectopic bone formation [5] and the role of BMPs in inducing osteoblast differentiation has been described for several BMPs.[6, 7] BMPs activate the type I and type II BMP receptor complexes, leading to initiation of signalling via phosphorylation of intracellular Smad proteins.[8] Activated Smads regulate expression of transcriptional factors and transcriptional co-activators important in osteoblast differentiation like Runx2 and Osterix.[7] Wnts are a family of secreted proteins that regulate many developmental processes, for example body axis formation, chondrogenesis and limb development.[9, 10] Canonical Wnt/β-catenin signalling has been shown to promote osteogenesis by stimulation of Runx2 gene expression.[11] In addition, activation of Wnt/β-catenin signalling promotes osteoblast cell proliferation and mineralization activity, reduces osteoblast apoptosis, and can suppress osteoclast differentiation induced by osteoblasts.[12] In the absence of Wnt activation, β-catenin is phosphorylated by glycogen synthase kinase 3β (GSK3β) in a complex with axin and adenomatous polyposis coli (APC), resulting in subsequent degradation. When Wnts bind to the Frizzled receptor and LRP5/6 co-receptor, axin is recruited to the membrane and the destruction complex is disrupted. Consequently, the phosphorylating action of GSK3β is prohibited and β-catenin accumulates in the cytoplasm, translocate into the nucleus and activates the transcription of Wnt target genes by binding to the TCF/LEF transcription complex.[13]

Hence, both Wnt and BMP signalling have important roles in promoting osteoblast differentiation and mineralization, and there are many reports showing an interaction between Wnt and BMP signalling.[14-18] Wnt signalling has been shown to increase BMP2 and BMP4 expression [19, 20] and on the other hand, Wnt1 and Wnt3a expression was increased by BMP2 [21], suggesting that both BMP and Wnt signalling may synergistically regulate each other. The activity of BMP and Wnt is also controlled by their intrinsic antagonists, which include noggin and sclerostin.[22, 23] Apart from natural produced inhibitors, many synthetic inhibitors have been developed to inhibit different aspects of the Wnt signalling pathway. One of these synthetic molecules is XAV939, a tankyrase inhibitor. Tankyrase marks axin for degradation, leading to disruption of the axin/APC/GSK3β complex. Thus, inhibition of tankyrase leads to accumulation of axin, breakdown of β-catenin and inhibition of the Wnt pathway.[24] PNU74654 binds to β-catenin, preventing it from binding to the TCF/LEF transcription complex and subsequently inhibits Wnt signalling.[25]

Sclerostin, produced by osteocytes, is an important regulator of bone formation and one of several known Wnt signalling inhibitors. Sclerostin inhibits canonical Wnt signalling in a similar manner as dickkopf-1 (Dkk-1) by binding to the LRP5/6 co-receptor.[26-28] Mutations in the gene encoding sclerostin, *SOST*, or the surrounding regulatory regions lead to sclerostin deficiency and bone overgrowth in sclerosteosis and van Buchem disease respectively.[29-32] In mice overexpressing *SOST* there is a significant reduction in osteoblast activity and subsequently bone formation.[30, 33] *In vitro* sclerostin inhibits the differentiation of pre-osteoblast cells.[28] Loss of sclerostin might prolong the bone formation phase of osteoblasts, resulting in the increase of bone mass. Sclerostin physiologically acts as a downstream molecule of BMP signalling to inhibit canonical Wnt signalling and negatively regulates bone mass.[23, 34]

The fact that sclerostin, a major regulator of bone formation through Wnt and BMP signalling, is limited to skeletal tissue and absence of sclerostin leads to an increase in bone formation, makes it an ideal drug target for bone formation. Recently it had been shown that treatment with romosozumab, a monoclonal antibody which binds to sclerostin, increases bone formation in patients suffering from osteoporosis.[35] BMPs are the most potent inducers of *SOST* expression and strong regulators of osteoblast differentiation.[36] Uncoupling of osteoblast differentiation regulators and their intrinsic inhibitors could possibly increase or prolong the BMP response, leading to more osteoblast differentiation and subsequent bone formation. Therefore the goal of this study was to investigate the effect of BMP and Wnt signalling on *SOST* expression and osteoblast differentiation.

Materials and methods

Cells, materials and reagents

The human osteosarcoma cell line SaOS-2 (ATCC, Manassas, VA, USA) was cultured in DMEM (Gibco, Carlsbad, CA, USA) supplemented with 10% Fetal Calf Serum (FCS; Greiner Bio One, Kremsmünster, Austria), 100U/ml penicillin and 100µg/ml streptomycin (Gibco). Murine mesenchymal progenitor cells KS483 were cultured in α-MEM without phenol red (Gibco) supplemented with FCS, penicillin and streptomycin and glutamax (Gibco).

Recombinant BMP4, Wnt3a and DKK1 were purchased from R&D Systems (Minneapolis, MN, USA). The specific GSK3β inhibitor 3-imidazo[1,2-a]pyridin-3-yl-4-(1,2,3,4-tetrahydro-[1,4] diazepino-[6,7,1-hi]indol-7-yl)pyrrole-2,5-dione (further referred to as GIN) was kindly provided by Dr. Rawadi (Prostrakan, France) and previously described by Engler et al. (2004) and Miclea et al. (2001).[15, 37] The Wnt signalling inhibitors XAV939 and PNU74654 were purchased at Sigma (St. Louis, MO, USA). The Wnt-responsive luciferase reporter BAT-luc has been described previously [15, 38] as is also the case for the BMP responsive element luciferase reporter BRE-luc.[39]

Luciferase experiments

SaOS-2 cells were seeded in 96-well plates at a density of 21,000 cells/cm^2 and cultured overnight to 70-80% confluence. The cells were transfected with BAT-luc or BRE-luc reporter construct and a pGL4-CAG renilla luciferase construct using FuGene HD transfection reagent (Promega Fitchburg WI USA) according to the manufacturer's instructions. After 24 hours of treatment with the indicated reagents, luciferase activity was determined using the Dual-Glo Luciferase assay system (Promega, Fitchburg, WI, USA) with a SpectraMax L luminometer (Molecular Devices, Sunnyvale, CA, USA). Relative luminescence was calculated as luciferase/ renilla luciferase and expressed as fold change versus control.

Differentiation experiments

KS483 cells were seeded at a density of 9,210 cells/cm^2. Every 3 to 4 days, the medium was changed. At confluence (from day 4 of culture onwards), ascorbic acid (50 µg/ml. Merck Inc., NY, USA) was added to the culture medium. When nodules appeared (from day 11 of culture onwards) β-glycerophosphate (5 mM; Sigma) was added. Every 3 to 4 days, medium samples (25ul) were analysed for alkaline phosphatase (ALP) activity by adding 200 µl of 2 mg/ml p-nitrophenylphosphate (Sigma) in 100 mM glycine/ 1 mM $MgCl_2$/ 0.1 mM $ZnCl_2$ buffer (pH 10.5) and reading for 10 min using a VERSAmax Tunable Microplate Reader (Molecular Devices) at 405 nm. ALP activity was determined as the slope of the kinetic measurement (mOD/min). Mineralization of the cultures was quantified using the fluorescent dye Bonetag as described previously.[40] Briefly, cells were incubated with 2 nM Bonetag 800 (Perkin Elmer) for 24 hours, washed with phosphate-buffered saline (PBS) and fixed with 3.7% buffered formaldehyde. The fixed cells were scanned with the Odyssey Infrared Imaging System (Li-COR) at a resolution of 42 µm, medium quality and intensity 5.0-6.5. Integrated intensity (counts/mm^2) of each well was calculated by the Odyssey software.

Quantitative RT-PCR and primers

Total RNA was isolated from SaOS-2 and KS483 cells using TriPure Isolation Reagent (Roche, Penzberg, Germany) 24 hours (SaOS-2 cells) or 8-10days (KS483 cells) after treatment with indicated reagents, respectively. cDNA was synthesized using M-MLV reverse transcriptase (Promega) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the Quantitect SYBRgreen PCR kit (Qiagen, Venlo, the Netherlands) with an iQ5 PCR cycler (BioRad, Hercules, CA, USA). For used primer sets (Eurogentec, Seraing, Belgium) see Table 1. β2-Microglobulin (β2M) was used as an internal control. Measurements were performed in triplicate and analysed using the ΔΔCt method.[41]

Table 1: Oligonucleotides used in RT-PCR

Gene		Forward	Reverse
β2M	Human	5'-TGCTGTCTCCATGTTTGATGTATCT-3'	5'-TCTCTGCTCCCCACCTCTAAGT-3'
	Murine	5'-TGACCGGCTTGTATGCTATC-3'	5'-CAGTGTGAGCCAGGATATAG-3'
SOST	Human	5'-TGCTGGTACACACAGCCTTC-3'	5'-GTCACGTAGCGGGTGAAGTG-3
	Murine	5'-TCCTCCTGAGAACAACCAGAC-3	5'-TGTCAGGAAGCGGGTGTAGTG-3'

Statistical analysis

Values represent mean ± SD. Differences were tested by one-way analysis of variance (ANOVA) followed by Bonferroni post hoc test using Graphpad Prism 5 software (La Jolla, CA, USA). Results were considered significant at $p < 0.05$.

Results

To address the interaction between Wnt and BMP signalling, different combinations of BMP4, Wnt3a or the GSK3β inhibitor (GIN) were added to SaOS-2 cells. Unfortunately we were unable to efficiently transfect KS483cells; therefore all transfection experiments were performed in SaOS-2 cells. The effect on BMP signalling was investigated using a BMP-responsive element driving luciferase expression, further referred to as BRE-luc. BMP4 alone dose-dependently increased the BRE-luc activity in SaOS-2 cells (Figure 1A). Although Wnt3a alone did not induce BRE-luc activity, it significantly increased BMP4-induced luciferase activity (Figure 1B). The BMP4-induced luciferase activity increased more than 5-fold in combination with 10^{-8}M GIN, even though GIN alone did not have any effect on the reporter (Figure 1C).

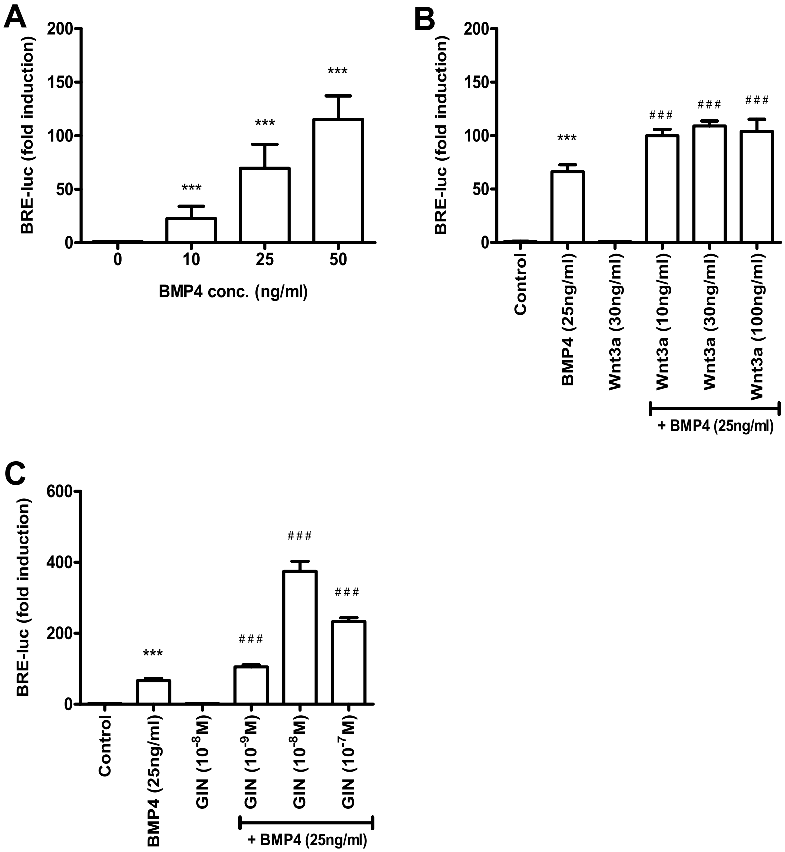

Figure 1: BMP reporter BRE-luc activity after stimulation with BMP4, Wnt3a and GIN. SaOS-2 cells were transfected with the BMP reporter construct BRE-luc and were stimulated with the indicated concentrations for 24 hours. Luciferase (n = 6) was measured, values represent mean ± SD. BRE-luc activity increased dose-dependently with BMP4 (A). Combined Wnt3a and BMP4 increased BRE-luc activation (B). GIN was more potent in increasing BMP4 induced BRE-luc activation (C). *** $p < 0.001$ compared to control. ### $p<0.001$ compared to BMP4.

The influence of the interaction between Wnt and BMP on Wnt signalling was investigated using the Wnt-responsive BAT-luc reporter. We observed a dose-dependent increase of BAT-luc activity after Wnt3a or GIN stimulation (Figure 2A,C). However, GIN was more potent in inducing BAT-luc activation, stimulating activity more than 400-fold compared to control at 10^{-8}M (Figure 2C). The GIN-induced BAT-luc activity increased more than 4-fold when combined with BMP4, even though BMP4 alone was not able to induce BAT-luc activity (Figure 2D). The Wnt-induced Bat-luc activity was not significantly increased in combination with BMP4 (Figure 2B).

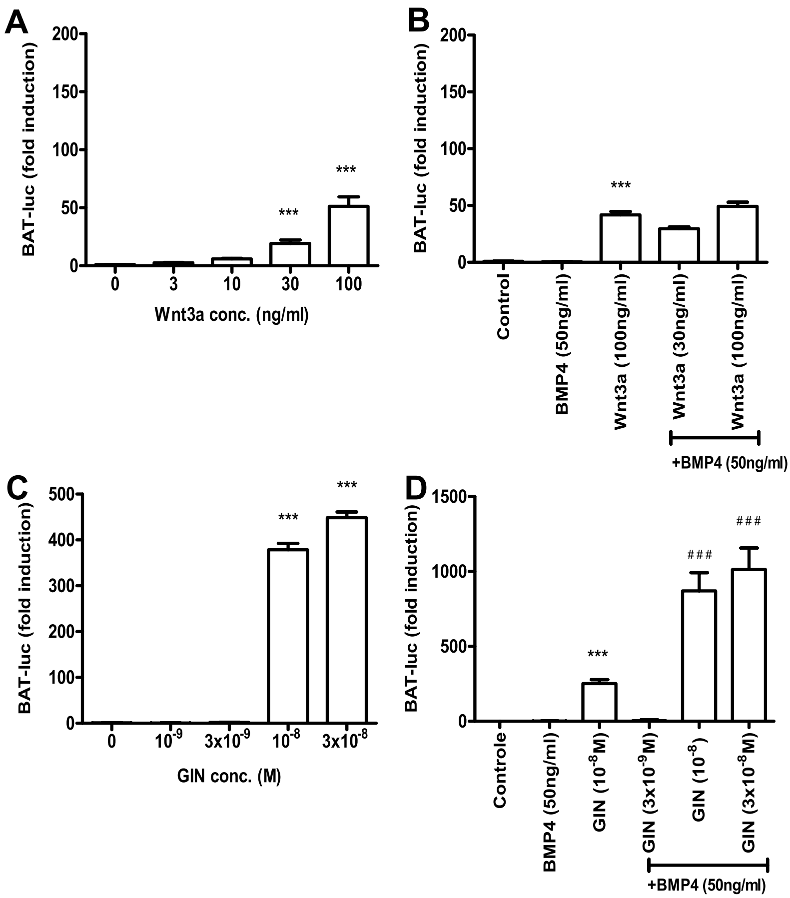

Figure 2: Wnt reporter BAT-luc activity after stimulation with Wnt3a, GIN and BMP4. SaOS-2 cells were transfected with the Wnt reporter construct BAT-luc and were stimulated with the indicated concentrations for 24 hours. Luciferase (n = 6) was measured, values represent mean ± SD. BAT-luc activity increased dose-dependently with Wnt3a (A) and GIN (C). Combined Wnt3a and BMP4 did not increase Wnt3a-induced BAT-luc activity (B), while combined GIN and BMP4 increased BAT-luc activity significantly (D). *** $p < 0.001$ compared to control. ### $p<0.001$ compared to GIN 10^{-8}M.

To address the interaction between Wnt and BMP signalling on *SOST* expression we used SaOS-2 cells, since these cells can express constitutively levels of mRNA *SOST* and therefore are a good model for studying the effect of GIN and BMP4 on *SOST* expression [42]. GIN dose-dependently decreased *SOST* expression levels. Even at a concentration of 3 x 10^{-9}M GIN, which showed no effect on BAT-luc activity, *SOST* expression was decreased (Figure 3A). Wnt3a was able to significantly decrease *SOST* expression only at a high concentration (100 ng/ml) (Figure 3B). Although BMP4 induced *SOST* expression (Figure 3C), a combination of BMP4 and GIN significantly decreased *SOST* expression (Figure 3C). When Wnt3a was added to BMP4-stimulated cells only a slight decrease in expression of *SOST* was observed (Figure 3C).

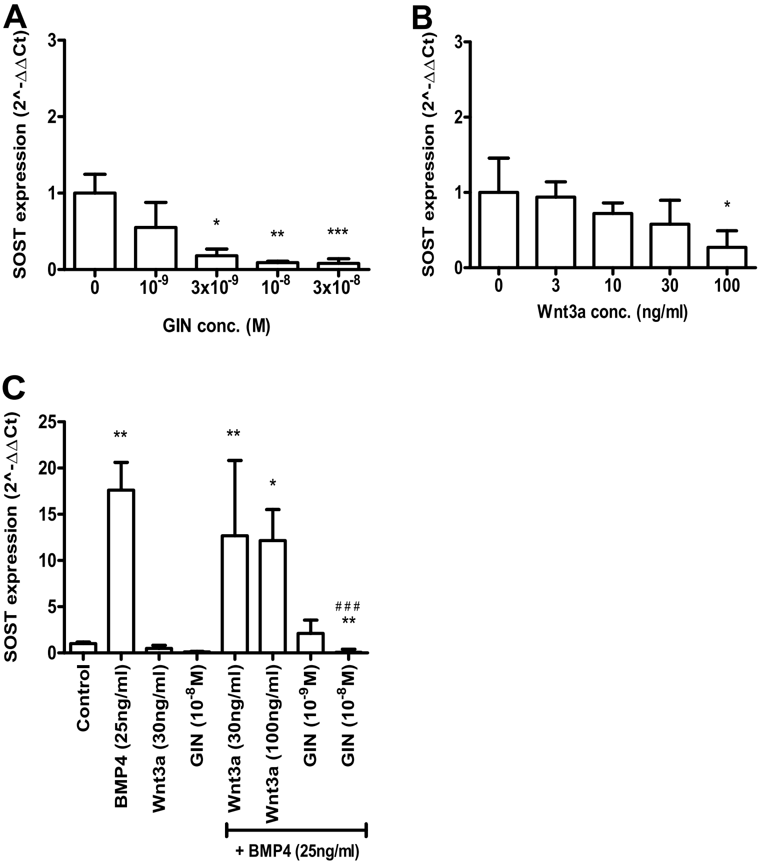

Figure 3: *SOST* mRNA expression (n=3) after stimulation with Wnt3a, GIN and BMP4. Values represent mean ± 95% CI. *SOST* expression decreased dose-dependently with GIN (A) and Wnt3a (B). Wnt3a did not decrease BMP4-induced *SOST* expression, while GIN even decreased BMP4-induced *SOST* expression below control levels (C). *p < 0.05 compared to control. ** p < 0.01 compared to control. *** p < 0.001 compared to control. ### p < 0.001 compared to BMP4.

Next, we investigated whether the down regulation of *SOST* expression observed after stimulation with GIN was a direct effect of Wnt/β-catenin signalling. To this purpose, three inhibitors of the Wnt signalling pathway were tested for their ability to counteract the effect of GIN on *SOST* expression. The extracellular inhibitor DKK1 could not inhibit Wnt signalling after stimulation with GIN (Figure 4A), whereas XAV939 and PNU74654 significantly inhibited the BAT-luc reporter activity (Figure 4A). However, neither of these inhibitors was able to restore *SOST* expression (Figure 4B).

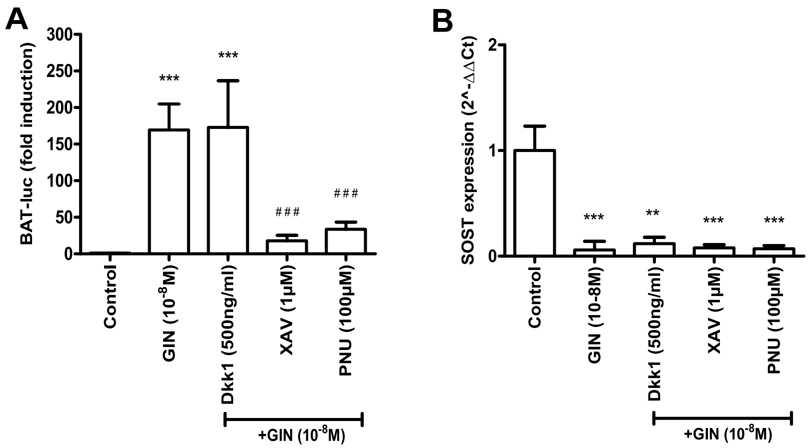

Figure 4: Wnt reporter BAT-luc activity and *SOST* expression after incubation with GIN and different Wnt signalling inhibitors. (A) Wnt reporter BAT-luc activity in SaOS-2 cells after stimulation with GIN (n = 6). Values represents mean ± SD. The extracellular inhibitor DKK1 could not inhibit GIN-induced BAT-luc activity. Both XAV939 and PNU74654 significantly inhibited BAT-luc activity. B) RNA from SaOS-2 cells was isolated after 24 hours incubation with GIN and other Wnt signalling inhibitors (n = 3). Values represent mean ± 95% CI. None of the inhibitors could restore *SOST* expression to control levels.** $p < 0.01$ compared to control, *** $p < 0.001$ compared to control, ### $p < 0.001$ compared to GIN control

Finally, we assessed the biological effect of down regulation of *SOST* expression by GIN in KS483 cells. This cell line provides a well-established model for investigating the process of osteoblast differentiation, rather than SaOS-2 cells, which represent human osteogenic osteosarcoma cells with late osteoblast characteristics [43, 44]. Since *SOST* mRNA expression is restricted after the onset of mineralization in osteoblastic cultures [45], we investigated the effect of GIN on *SOST* mRNA expression during the first days of mineralization (e.g. after 13 or 14 days of culture). Osteogenic differentiation of the cultures was monitored by measuring alkaline phosphatase activity in the medium and matrix mineralization. As shown in Figure 5A, addition of BMP4 significantly increased ALP activity on day 7, 11 and 14. Addition of GIN even further increased BMP4-increased ALP activity. Treatment of the cells with BMP4 resulted in an increase of mineralization, while addition of GIN increased BMP4-induced mineralization even further (Figure 5B,D). Consistent with the increase in mineralization by BMP4, *SOST* mRNA expression was also increased by BMP4 (Figure 5C). GIN alone has a slight but not significant inhibitory effect on both mineralization and *SOST* expression (Figure 5B,C). However, when GIN was added in combination with BMP4, *SOST* mRNA expression was reduced (Figure 5C).

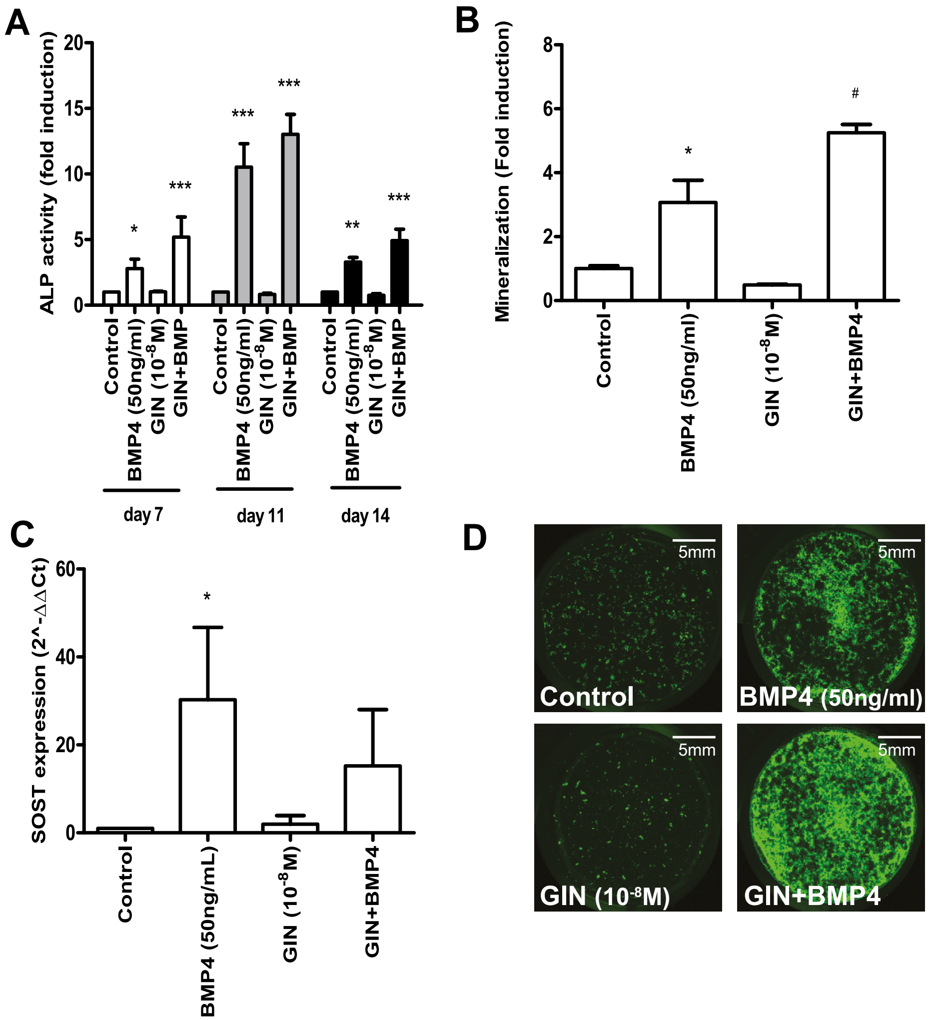

Figure 5: Osteoblast differentiation of KS483 cells after stimulation with BMP4 and GIN. (A) Alkaline Phosphatase (ALP) activity in medium. BMP4 increased ALP activity during differentiation, while GIN increased the BMP4 induced ALP activity even further. (B+D) Mineralization measured by Bonetag. BMP4 increased mineralization, while GIN slightly inhibited mineralization. A combination of BMP4 and GIN increased the mineralization significantly. (C) *SOST* mRNA expression (n = 4). BMP4 increased *SOST* expression, while GIN slightly decreased BMP4 induced *SOST* expression. Values represent mean ± SD of four independent experiments, except for (D) which represents pictures from one representative experiment. ** $p < 0.01$ compared to control, *** $p < 0.001$ compared to control, ## $p < 0.01$ compared to BMP4.

Discussion

Both BMP and Wnt signalling have been shown to play important roles in promoting osteoblast differentiation and mineralization.[7, 12] Interaction between both signalling pathways has been found in several studies, suggesting that both BMP and Wnt signalling may synergistically regulate osteoblast differentiation.[14-18] Since BMP and Wnt signalling induce their intrinsic antagonists [23] and *SOST* seems to be involved in both pathways, we investigated the effect of these pathways on *SOST* expression, both separately and in combination.

SaOS-2 cells represent human osteogenic osteosarcoma cells with late osteoblast characteristics, transitioning towards osteocytes.[44] These cells belong to one of the few cell lines constitutively expressing *SOST*, are easy to transfect and are therefore used for studying signalling pathways and the influence on *SOST* expression. However, this cell line is less appropriate to investigate the process of osteoblast differentiation. Therefore, we used KS483 cells, a murine mesenchymal progenitor cell line, which represents a more accurate model to study effects on osteoblast differentiation.[43]

Our results in SaOS-2 cells show that inhibition of GSK3β, either via the Wnt pathway by stimulation with Wnt3a or by direct inhibition using the GSK3β inhibitor GIN, resulted in a decreased expression of the Wnt signalling inhibitor *SOST*. Interestingly, GIN was much more potent in the down regulation of *SOST*. Moreover, BMP4-induced *SOST* expression was decreased by GIN, but not by Wnt3a.We suggest this is the result of the more potent induction of the Wnt pathway by GIN compared to Wnt3a, as was shown by a much higher induction of BAT-Luc by GIN. In addition, when Wnt3a or GIN was combined with BMP4, both Wnt as well as BMP signalling were further increased, suggesting a synergistic mechanism. Again, GIN was much more potent in inducing both pathways in combination with BMP4.

Because of its clear connection to regulation of bone cells, canonical Wnt signalling seems the most plausible pathway involved in the down regulation of *SOST*. However, *SOST* expression was also decreased at a concentration of GIN where no increase in BAT-luc activity was seen. Moreover, the β-catenin binding inhibitor PNU74654 was not able to restore *SOST* expression after treatment with GIN. This suggests that down regulation of *SOST* by GIN is not a direct effect of the canonical Wnt pathway, but appears to be mediated independent of β-catenin. Although GIN was thoroughly screened for selectivity against a panel of kinases [37], further experiments are needed to exclude cross-reactivity or off-target effects of GIN. In addition, we found a connection between GSK3β inhibition and BMP signalling on the down regulation of *SOST*. Therefore, we can rule out the involvement of solely canonical Wnt signalling in the regulation of *SOST*. Previous studies already described a mechanism in which GSK3β phosphorylation primes Smad1 for ubiquitination and degradation. With this mechanism GSK3β controls the duration of Smad1 activation and therefore BMP signalling.[46, 47] A similar mechanism may be true for the duration of

Smad6 and Smad7 activation, which have been shown to inhibit *SOST* promoter activity.[48]

The combined effect of GSK3β inhibition and BMP on *SOST* expression observed in SaOS-2 cells was also observed in KS483 cells. The biological effect of downregulation of *SOST* by GIN was measured by alkaline phosphatase (ALP) activity in the medium and mineralization of the matrix in KS483cells. A combination of GIN and BMP4 increased osteoblast differentiation. Our results are in line with Fukuda et al. (2010) who have shown that BMP4 and canonical Wnt cooperatively induced osteoblast differentiation through a GSK3β-dependent and β-catenin independent mechanism.[18] Although the decrease in BMP4-induced *SOST* expression by GIN in our experiments was not statistically significant, we hypothesize that the increase in osteoblast differentiation is due to the uncoupling of BMP signalling and *SOST* expression.

In conclusion, this study showed that uncoupling of BMP signalling and *SOST* expression could increase BMP-induced osteoblast differentiation. Furthermore, our results propose the existence of a new regulatory pathway for expression of *SOST*, which is mediated by GSK3β but independent of β-catenin. Further studies are necessary to identify the exact mechanism of regulating sclerostin via GSK3β and the way it interacts with other pathways during bone metabolism.

Inhibition of sclerostin has interesting clinical applications. Recently, a monoclonal antibody inhibiting sclerostin has been shown to enhance bone formation and to prevent implant loosening in preclinical studies [49, 50] and is currently tested in clinical trials phase III (ClinicalTrials.gov number, NCT01631214).[35] Another approach of inhibiting sclerostin and subsequently increasing bone mass would be via GSK3β inhibition as shown in our study. However, we propose that this approach of inhibiting sclerostin would be suitable for local applications only, since a study of Miclea et al. (2011) showed that systemic treatment with GIN induced osteoarthritis-like features in mice.[15] For example, local inhibition of sclerostin via GSK3β could have advantages in fracture healing or could improve osseointegration of implants by local increase of bone growth.

Acknowledgements

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PERI-PROSTHETIC TISSUE CELLS SHOW OSTEOGENIC CAPACITY TO DIFFERENTIATE INTO THE OSTEOBLASTIC LINEAGE

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Abstract

During the process of aseptic loosening of prostheses, particulate wear debris induces a continuous inflammatory-like response resulting in the formation of a layer of fibrous peri-prosthetic tissue at the bone-prosthesis interface. The current treatment for loosening is revision surgery which is associated with a high morbidity rate, especially in old patients. Therefore, less invasive alternatives are necessary. One approach could be to re-establish osseointegration of the prosthesis by inducing osteoblast differentiation in the peri-prosthetic tissue. Therefore, the aim of this study was to investigate the capacity of peri-prosthetic tissue cells to differentiate into the osteoblast lineage. Cells isolated from peri-prosthetic tissue samples (n=22) – obtained during revision surgeries – were cultured under normal and several osteogenic culture conditions. Osteogenic differentiation was assessed by measurement of Alkaline Phosphatse (ALP), mineralization of the matrix and expression of several osteogenic genes. Cells cultured in osteogenic medium showed a significant increase in ALP staining ($p=0.024$), mineralization of the matrix ($p<0.001$) and ALP gene expression ($p=0.014$) compared to normal culture medium. Addition of bone morphogenetic proteins (BMPs), a specific GSK3β inhibitor (GIN) or a combination of BMP and GIN to osteogenic medium could not increase ALP staining, mineralization and ALP gene expression. In one donor, addition of GIN was required to induce mineralization of the matrix. Overall, we observed a high inter-donor variability in response to osteogenic stimuli. In conclusion, peri-prosthetic tissue cells, cultured under osteogenic conditions, can produce alkaline phosphatase and mineralized matrix and therefore show characteristics of differentiation into the osteoblastic lineage.

Keywords: Aseptic loosening, peri-prosthetic tissue, osteogenic differentiation, regenerative medicine, total hip revision.

Introduction

Aseptic loosening is the most common long-term cause of failure in total joint arthroplasty.[1] The process of loosening is thought to be a complex interplay between mechanical as well as biological factors. Particulate wear debris, continuously generated by articulating motion at the bearing surfaces, has been implicated as one of the primary causes initiating peri-prosthetic bone loss.[2] Subsequently, a fibrous-like peri-prosthetic tissue layer with poor mechanical properties is formed, triggering bone resorption and prosthesis displacement.[3]

The current treatment for aseptically loosened prostheses is invasive revision surgery, which consists of removal of the old prosthesis together with the peri-prosthetic tissue, and insertion of a new prosthesis. Sometimes, in addition, bone augmentation has to be done with allograft bone, depending on the severity of bone loss. This procedure is highly demanding for the patient and is associated with blood loss, complications, and morbidity, especially in elderly patients with a poor general health condition.[4-6] Furthermore, revision prostheses display poorer clinical and functional performance than that of the primary prosthesis.[7-9] Therefore, less demanding therapies alternative to extensive revision surgery but with adequate functional performance are necessary. Currently, several minimally invasive techniques are being investigated to remove the peri-prosthetic tissue and stabilize the loosened prosthesis by subsequent bone cement injection.[10, 11]

Another approach would be to promote bone formation in the peri-prosthetic tissue in order to compensate peri-prosthetic bone loss and subsequently stabilize the loosened prosthesis. This could be accomplished in a minimally invasive way by percutaneous, local introduction of osteogenic factors at the peri-prosthetic space, which will drive osteogenic differentiation of peri-prosthetic tissue cells.

However, little is known about the role of peri-prosthetic tissue cells in bone formation. Most effort so far has been concentrated on understanding the role of these cells in bone resorption around the implant. The few studies investigating their role in bone formation show that peri-prosthetic tissue cells produce factors that suppress osteoblast function and induce production of inflammatory cytokines.[12, 13] Moreover, wear particles and metal ions can directly affect osteoblasts by reducing type 1 collagen production[14, 15] and decreasing alkaline phosphatase activity as well as calcium deposition.[16] In addition, wear particles have been shown to decrease osteoblast proliferation[15, 17], change the phenotype of mature osteoblasts[18], and stimulate osteoblasts to secrete inflammatory cytokines.[14-16, 19] Remarkably, one study revealed that cells from the peri-prosthetic tissue produce several osteoblastic proteins themselves.[20] In agreement, histological assessment of peri-prosthetic tissue demonstrated that this tissue exhibits osteogenic characteristics as shown by the presence of intramembranous formation of osteoid[21, 22], an increased mineral apposition rate and bone formation rate with active osteoblast lining and production of immature bone matrices with poor bone quality.[22] Furthermore, an increased expression of several bone morphogenetic proteins (BMPs) in several cell types of the peri-prosthetic tissue was found.[23] As BMPs are regulators and potent inducers of osteoblast differentiation[24], the local increase of BMP synthesis in peri-prosthetic tissue could be an attempt to regenerate or maintain implant fixation. However, to our knowledge, nothing is known about the capability of peri-prosthetic tissue cells to (re)generate bone themselves. Therefore, the aim of this study is to investigate whether cells within the peri-prosthetic tissue are able to differentiate into the osteoblastic lineage.

Materials and methods

Peri-prosthetic tissue samples

Peri-prosthetic tissue samples harvested from aseptically loosened femoral stems or acetabular components of 22 patients were obtained during revision surgery of total hip replacements. The peri-prosthetic tissue was collected as "waste" material and as such should not be traceable to specific patients according to Dutch Medical Ethics laws and legislation. Therefore, only limited donor characteristics are available (see Table 1). Collected samples were kept in sterile NaCl 0.9% at 4°C, for a maximum of 24 hours, until they were processed. This study was approved by the Medical Ethics Committee of Leiden University Medical Center (C12-107).

Cell culture

Collected tissue samples were minced and incubated at 37°C for two hours in α-MEM (Gibco, Carlsbad, CA, USA) with collagenase I A (2mg/mL; Sigma-Aldrich, St Louis, MO, USA). The cell suspension was then centrifuged and washed twice in α-MEM supplemented with 10% Fetal Calf Serum (FCS; Greiner Bio One, Kremsmünster, Austria). Cells were cultured in petri-dishes in α-MEM supplemented with 10% FCS, 1% Glutamax (Gibco), 3% penicillin and streptomycin (Gibco) and 25µg/ml Amphotericin B (Gibco) for 72 hours. Thereafter, cells were cultured in the same medium but without Amphotericin B. When cultures reached 90% confluence, the cells were transferred to 75cm^2 flasks. For the experiments cells from passage 1 or 2 were used.

The human osteosarcoma cell line SaOS-2 (ATCC, Manassas, VA, USA) and the human dermal fibroblast cell line (HDF-a; ScienCell, Carlsbad, CA, USA) were cultured in DMEM (Gibco) supplemented with 10% FCS and 1% penicillin and streptomycin (Gibco). These cell lines were used as positive and negative controls in the experiments, respectively.

Table 1: Demographic data from included peri-prosthetic tissue samples.

Donor	Sex	Age	Fixation	Bearing	Cup/Stem	Years in situ	Visible presence of wear debris
1	F	81	Cemented	Metal/PE	Cup	>5	Yes
2	M	74	Cementless	Metal/PE	Cup	>5	No
3	F	85	Cemented	Metal/PE	Cup	>5	Yes
4	M	59	Cemented	Metal/PE	Stem	>5	Yes
5	M	81	Cemented	Metal/PE	Cup	>5	No
6	F	79	Cemented	Metal/PE	Stem	>5	Yes
7	M	55	Cementless	Metal/Metal	Cup	>5	Yes
8	F	80	Cemented	Metal/PE	Cup	>5	No
9	F	73	Cementless	N/A	N/A	>5	Yes
10	F	75	Cementless	Ceramic/PE	N/A	>5	No
11	F	N/A	Cemented	Metal/PE	Cup	>5	Yes
12	M	79	Cemented	Metal/PE	Cup+Stem	>5	No
13	F	80	Cementless	Metal/PE	Cup	>5	No
14	F	86	Cemented	Ceramic/PE	Cup	>5	Yes
15	M	32	Cemented	Metal/PE	Cup	2-5	Yes
16	F	76	Cementless	Ceramic/PE	Cup	>5	No
17	F	74	Cemented	Metal/PE	Cup+Stem	>5	No
18	M	69	Cemented	Metal/PE	N/A	>5	Yes
19	M	84	Cemented	Metal/PE	Stem	>5	Yes
20	F	71	Cementless	Metal/PE	N/A	>5	No
21	M	66	Cemented	Metal/PE	Cup	>5	Yes
22	F	80	Cemented	Metal/PE	Stem	2-5	Yes

F = female, M = Male, PE = Polyethylene, N/A = not available

Osteogenic differentiation experiments

Peri-prosthetic tissue cells were seeded at a density of 8,650 cells/cm^2. To induce osteoblast differentiation, normal culture medium (NM, as described above) was supplemented with freshly added ascorbic acid (50µg/ml; Merck Inc., NY, USA), β-glycerophosphate (5mM; Sigma-Aldrich) and dexamethasone (0.1µM; Sigma-Aldrich) with or without recombinant human BMP-2, BMP- 6 (50ng/mL; R&D Systems, Minneapolis, MN, USA) or a specific GSK3β inhibitor (GIN; 10nM; kindly provided by Dr. Rawadi, Prostrakan, France; Engler et al.[25]) Cell cultures were subjected randomly to either BMP-2 or BMP-6. The culture medium was replaced every 3 to 4 days.

Alkaline Phosphatase activity

Alkaline Phosphatase (ALP) activity was assessed by both histochemical staining and a colorimetric assay. For ALP staining, cells were cultured for 18/21 days after which they were fixed in 3.7% buffered formaldehyde and stained with a solution containing 0.1mg/ml naphthol ASMX phosphate (Sigma), 0.5% N, N- dimethylformamide, 2mM $MgCl_2$, and 0.6mg/ml of fast blue BB salt (Sigma) in 0.1mM Tris–HCl (pH 8.5) for 5 minutes. Thereafter, ALP staining was completely washed out of the cell layer with a freshly prepared solution of 50mM NaOH in EtOH. The absorbance was measured at 500nM using a VERSAmax Tunable Microplate Reader (Molecular Devices). Enzymatic ALP activity was measured after 4, 6, 13, 18, 21, 25, 28 and 32 days of culture using p-nitrophenyl phosphate (pNPP) as described by van der Horst et al. 2002.[26] Briefly, the cells were lysed and ALP activity was measured kinetically using 6 mmol/L pNPP at 405nm using a VERSAmax Tunable Microplate Reader. DNA concentration in the cell lysate was measured using the fluorescent dye bisBenzimide H 33258 (Hoechst 33258, Sigma) and was calibrated against a DNA standard (0.5–10µg/mL herring sperm DNA). ALP activity was corrected for the amount of DNA in the culture.

Mineralization

Mineralization of the cultures was assessed using the fluorescent dye Bonetag (Li-COR Biosciences, Lincoln, NE, USA) as described previously.[27] Briefly, 24 hours before fixation with 3.7% buffered formaldehyde, 2nM Bonetag 800 was added to the culture medium. The fixed cells were scanned with the Odyssey Infrared Imaging System (Li-COR) at a resolution of 42µm, intensity 6.5 and medium quality. Integrated intensity (counts/mm^2) of each well was calculated by the Odyssey software.

Quantitative RT-PCR

Total RNA was isolated from cells using RNA-Bee (Tel-Test Inc., Friendswood, TX, USA). cDNA was synthesized using M-MLV reverse transcriptase (Promega, Fitchburg WI USA) according to the manufacturer's instructions. Quantitative RT-PCR was performed using the Quantitect SYBRgreen PCR kit (Qiagen, Venlo, The Netherlands) with an iQ5 PCR cycler (BioRad, Hercules, CA, USA). For used primer sets, all spanning at least one intron, see Table 2. Data were normalized relative to GAPDH expression. Levels of gene expression in differentiation experiments were expressed as fold-change relative to expression in SaOS-2 and HDFA cells using the $2^{-\Delta\Delta Ct}$ method. Basal levels of gene expression at beginning of experiments were expressed as fold-change relative to expression in positive controls (e.g. SaOS-2 cells, HDFA cells, human endothelial cells and human monocytes).

Table 2: Oligonucleotides used in RT-PCR

Gene	Forward	Reverse
GAPDH	5'-GACAGTCAGCCGCATCTTC-'3	5'-GCAACAATATCCACTTTACCAGAG-'3
ALP	5'-TAAAGCAGGTCTTGGGGTGC-'3	5'-GGGTCTTTCTCTTTCTCTGGCA-'3
Col1a1	5'-TTTGGATGGTGCCAAGGGAG-'3	5'-CACCATCATTTCCACGAGCA-'3
OCN	5'-CCCAGCGGTGCAGAGTC-'3	5'-TCAGCCAACTCGTCACAGTC-'3
S100A4	5'-TTGGTTTGGTGCTTCTGAGATGT-'3	5'- TCACCCTCTTTGCCCGAGTA-'3
Vimentin	5'- CCAAACTTTTCCTCCCTGAACC-'3	5'- CGTGATGCTGAGAAGTTTCGTTGA-'3
Endoglin	5'- TCACCACAGCGGAAAAAGGT-'3	5'- CAGGAACTCGGAGACGGATG-'3
PECAM	5'- AGACGTGCAGTACACGGAAG-'3	5'- CTTTCCACGGCATCAGGGA-'3
CD68	5'- AGGCTGGCTGTGCTTTTCTC-'3	5'- TCTCTGTAACCGTGGGTGTC-'3

Statistical analysis

Statistical analysis was performed using SPSS (IBM SPSS Statistics 23). To account for treatment clustering within donors during osteogenic differentiation, linear mixed-effects modelling was used to analyse the ALP activity staining, mineralization and gene expression data, while two-way (mineralization) or three-way (ALP cell layer) ANOVA was used for the outcome measures in the time-dependent experiments.

Linear regression was performed for testing the relation between variability levels of ALP activity and/or mineralization and patient- and implant characteristics as well as between levels of mineralization and basal gene expression levels of "cell-specific" genes. For all tests, a p-value of <0.05 was regarded as statistically significant. Values represent mean ± SD, unless stated otherwise.

Results

The effect of osteogenic stimuli on osteogenic differentiation of peri-prosthetic tissue cells was studied in 22 donors after 18 to 21 days of culture. When cultured in normal medium (NM), cells displayed ALP activity (1A). Stimulation of cells with osteogenic medium (OM) significantly increased ALP activity (p=0.024). OM supplemented with either BMP-2, GIN and BMP-2+GIN or, alternatively, supplemented with BMP-6 and BMP-6+GIN did not significantly increase ALP activity (Figure 1B). In all culture conditions, variation in the level of ALP activity was observed between donors (Figure 1A, B). These variations between donors makes it impossible to assess differences between BMP-2 and BMP-6. Mineralization of the matrix was studied using the fluorescent dye Bonetag. When cells were cultured in NM, little to no fluorescence was observed, whereas OM induced mineralization in 15 out of 22 donors, although to (very) different extents (Figure 2A, B). In 5 out of 15 donors mineralization could be increased compared to OM when BMP, GIN or BMP+GIN was added (Supplementary Figure 1).

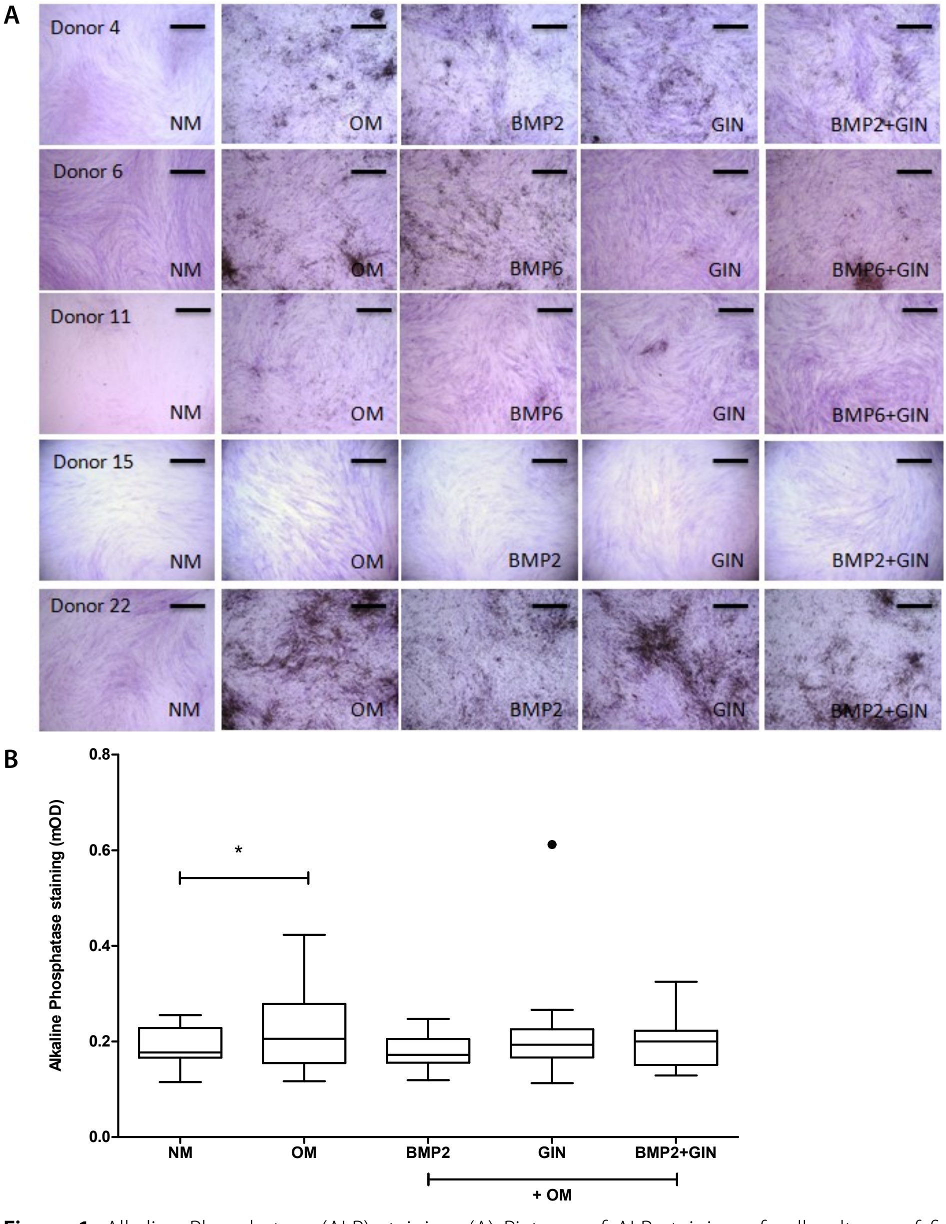


Figure 1: Alkaline Phosphatase (ALP) staining. (A) Pictures of ALP staining of cell cultures of five representative donors cultured in NM or stimulated with OM or OM supplemented with either BMP-2 or BMP-6, GIN and BMP-2 or BMP-6 + GIN. Bars represent 1 mm. (B) Boxplot showing the mOD after extraction of ALP staining from the cultures of twenty-two donors cultured in NM, OM or OM supplemented with either BMP-2, GIN or BMP-2 + GIN. Values represent mean ± SD, dots represents 1.5*IQR. NM: normal medium; OM: osteogenic medium; BMP: bone morphogenetic protein; GIN: GSK3β inhibitor; IQR: Inter Quartile Range.
* $p<0.05$ compared to NM.

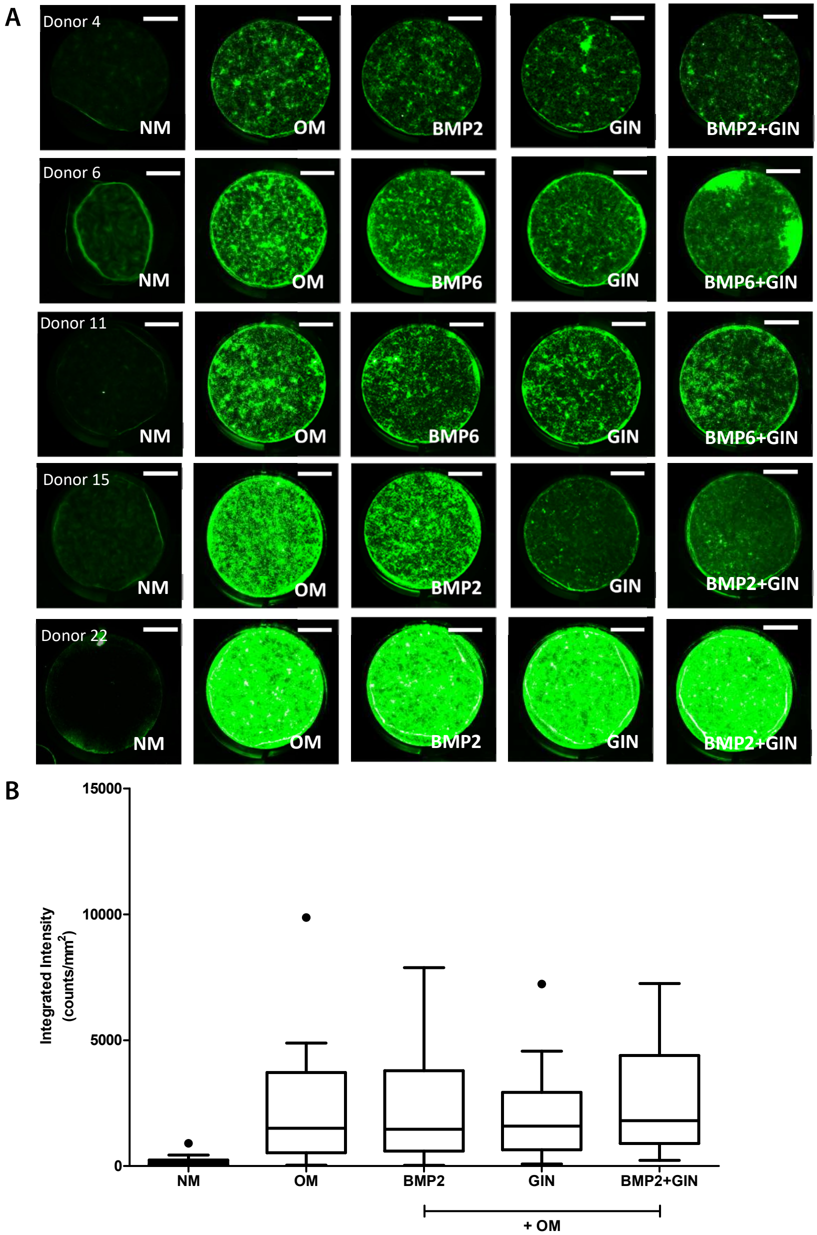


Figure 2: Mineralization of the matrix as measured by Bonetag (fluorescent dye). (A) Pictures of Bonetag fluorescent staining of cultures of five representative donors stimulated with OM or OM supplemented with BMP-2 or BMP-6, GIN and BMP-2 or BMP-6 + GIN. Normal culture medium served as a negative control. Bars represent 5 mm. (B) Boxplot showing the Integrated Intensity of the fluorescence of twenty-two donors stimulated with OM or OM supplemented with either BMP-2, GIN or BMP-2 + GIN. Values represent mean ± SD, dots represents 1.5*IQR. NM: normal medium; OM: osteogenic medium; BMP: bone morphogenetic protein; GIN: GSK3β inhibitor; IQR: Inter Quartile Range.

Interestingly, in donor 3, mineralization could not be induced by OM alone, but addition of GIN was needed to induce mineralization. Furthermore, in donor 19, mineralization could not be induced by OM alone nor OM with additives (Supplementary Figure 1). Overall, addition of BMP, GIN or BMP+GIN could not significantly increase mineralization compared to OM (Figure 2B). Subsequently, we also investigated the response to continuous stimulation with BMP-6. However, there were no significant differences in ALP activity and mineralization compared to single stimulation at day 4 (data not shown).

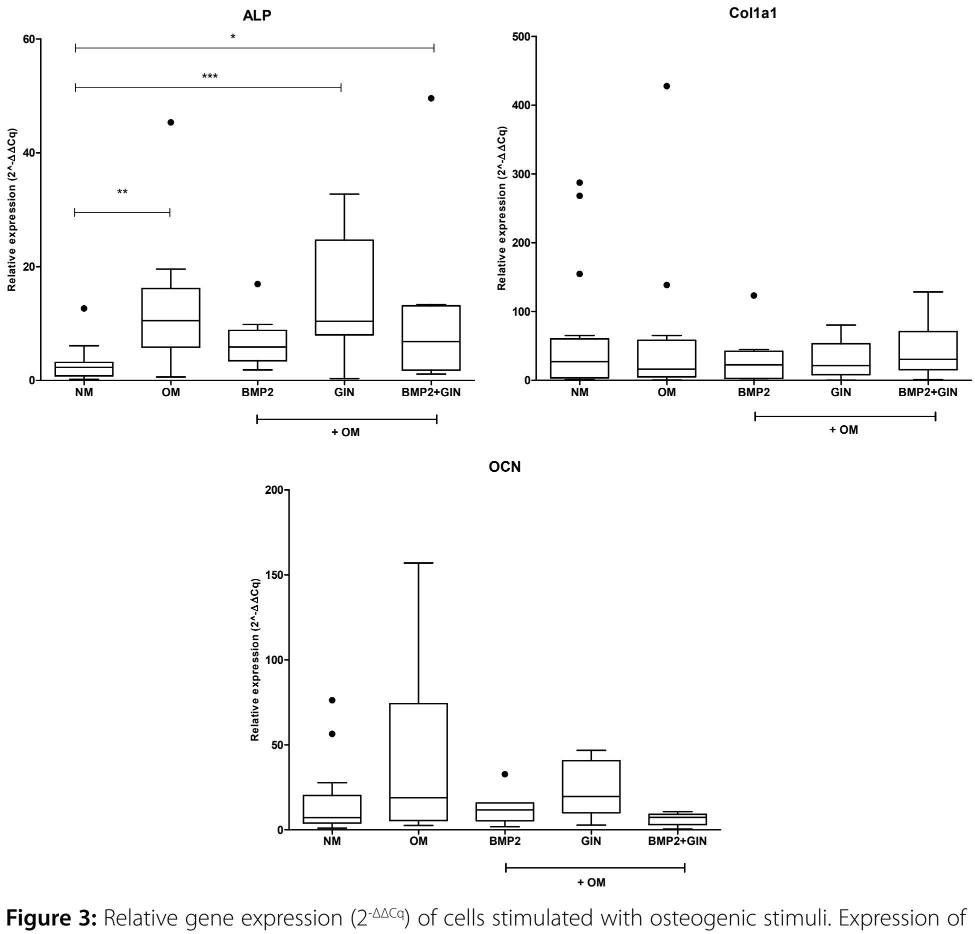


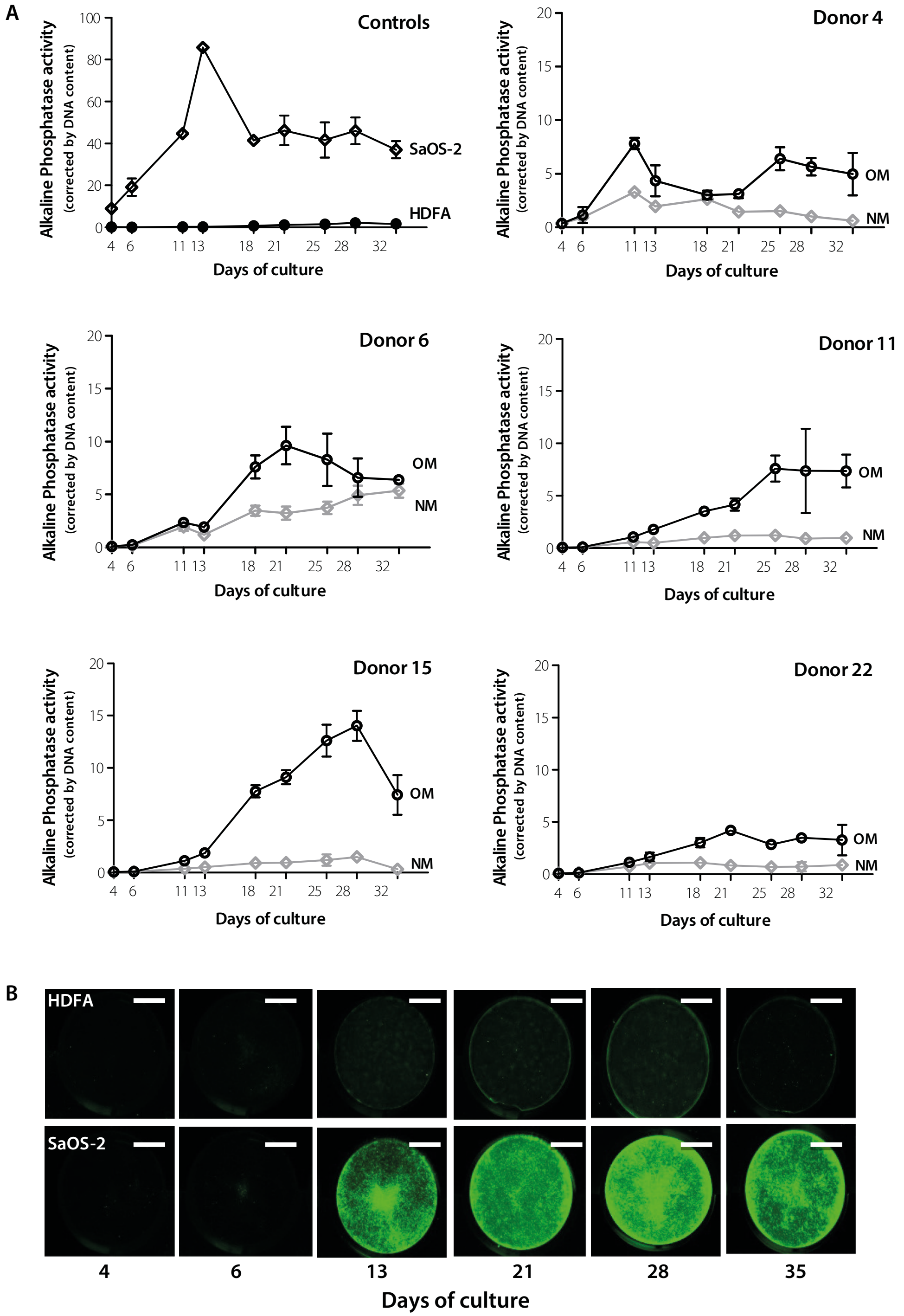
Figure 3: Relative gene expression ($2^{-\Delta\Delta Cq}$) of cells stimulated with osteogenic stimuli. Expression of alkaline phosphatase (ALP), alpha-1 type I collagen (Col1a1) and osteocalcin (OCN) was corrected for internal control GAPDH and the geometric mean of calibrators SaOS-2 and HDFA. Boxplot showing the mean ± SD, dots represent 1.5*IQR. NM: normal medium; OM: osteogenic medium; BMP: bone morphogenetic protein; GIN: GSK3β inhibitor; IQR: Inter Quartile Range.
*p<0.05, **p<0.01, ***p<0.001.

The effect of osteogenic stimuli on ALP, Alpha-1 type I collagen (Col1a1) and osteocalcin (OCN) gene expression was investigated in 19 donors (Figure 3). OM and OM supplemented with GIN or BMP-2+GIN could significantly increase ALP gene expression (resp. $p=0.014$, $p<0.001$, $p=0.023$) compared to NM. Col1a1 and OCN gene expression were not significantly different between culture conditions.

We investigated whether the observed variation in levels of ALP activity and mineralization between donors could be explained by patient- and implant characteristics. Type of fixation (ALP: $\beta=0.026$, $p=0.538$; Mineral: $\beta=-174.30$, $p=0.875$), gender (ALP: $\beta=0.06$, $p=0.187$; Mineral: $\beta=80.21$, $p=0.947$) or age (ALP: $\beta=0.001$, $p=0.693$; Mineral: $\beta=-21.14$, $p=0.661$) could not explain the found variations in ALP activity and mineralization.

Next, we tested whether variation observed in levels of ALP activity and mineralization between donors could be explained by degree of responsiveness to osteogenic stimuli. Since ALP activity and mineralization as described above were only measured at a single time-point (e.g. after 18-21 days of culture), variations observed in these markers for osteogenic differentiation could be explained by differences in response-rate / degree of responsiveness of the cells from different donors to osteogenic conditions. Therefore, in five donors, we investigated the response to osteogenic stimuli over time. Since there was almost no additional effect of BMPs and/or GIN compared to OM alone, we decided to investigate only the response to OM over time. In all donors, at each time-point, we observed a significant difference ($p<0.001$) between culture conditions in ALP activity in the cell layer (Figure 4A). For mineralization, all donors showed a significant different pattern in mineralization when cultured with OM, with mineralization observed from day 13, 21 or 28 onwards (Figure 4B, C). In all cases, mineralization occurred directly after a decrease in ALP activity was seen (Figure 4A).

Furthermore, we investigated whether the observed variation in osteogenic responsiveness could be explained by the heterogeneous composition of the cell population at the start of experiments. Therefore, we selected several 'cell-specific' genes to compare cell populations between donors. All samples showed the presence of macrophage, fibroblast, osteoblast and endothelial cells, however, a high inter-donor variability in basal gene expression levels of peri-prosthetic tissue was observed (Figure 5). No significant associations were found between levels of Integrated Intensity (mineralization at day 18-21) and gene expression levels of ALP ($\beta=-474.89$, $p=0.91$), OCN ($\beta=-72.15$, $p=0.115$), S100A4 ($\beta=-141.80$, $p=0.49$), Vimentin ($\beta=-562.25$, $p=0.72$), Endoglin ($\beta=-13287.76$, $p=0.194$), Pecam-1 ($\beta=-251941.78$, $p=0.067$) or CD68 ($\beta=-148.69$, $p=0.59$).


A
Controls
Alkaline Phosphatase activity
(corrected by DNA content)
100
80
60
40
20
0
SaOS-2
HDFA
4 6 11 13 18 21 25 28 32
Days of culture
Donor 4
20
15
10
5
0
OM
NM
Donor 6
Donor 11
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HDFA
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28
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Days of culture

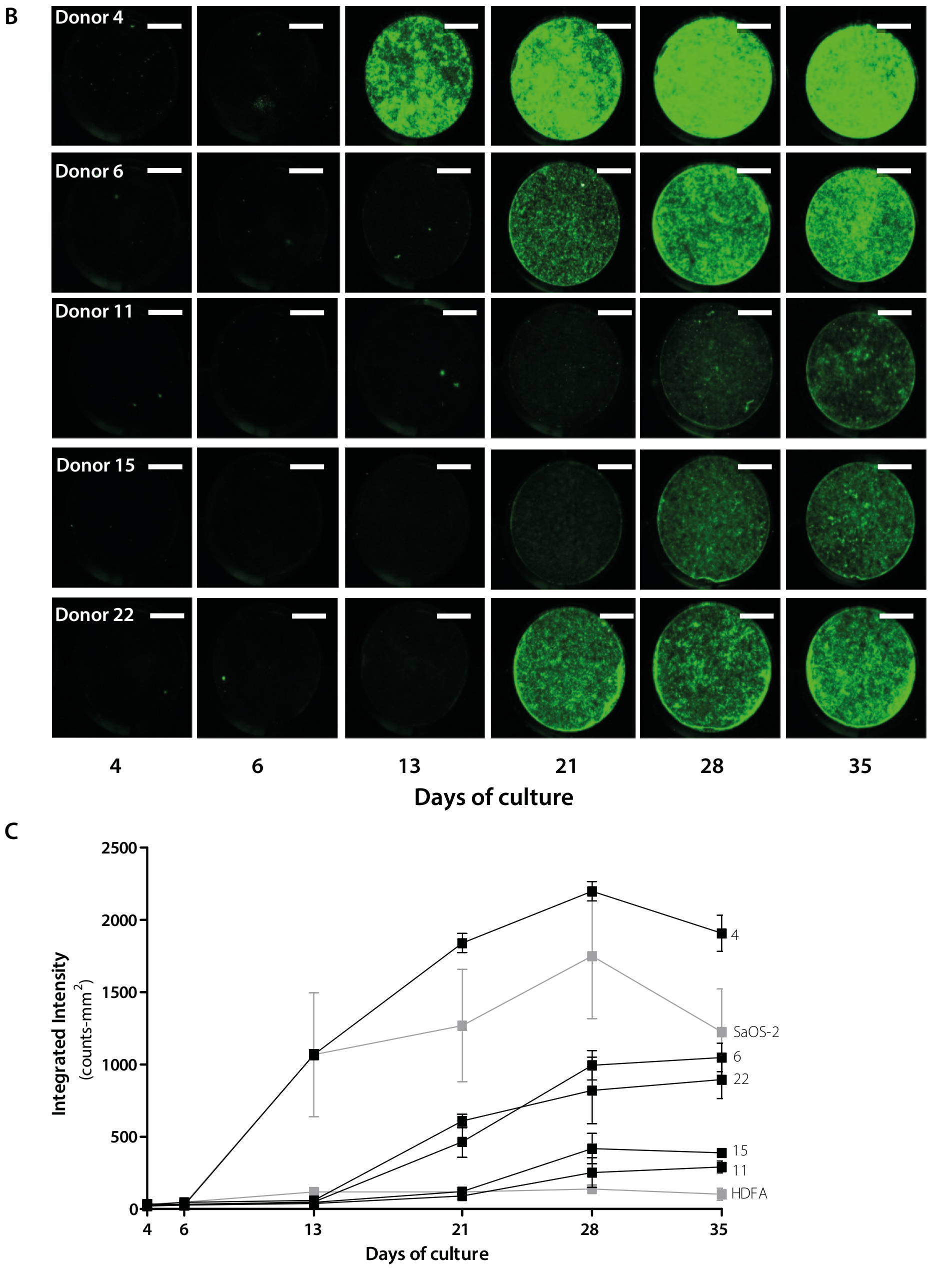
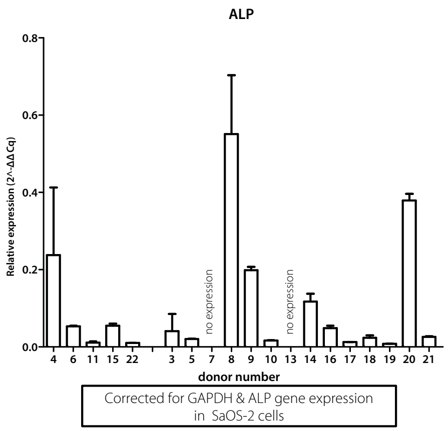
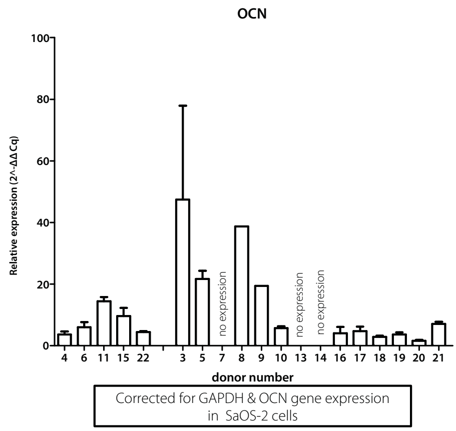
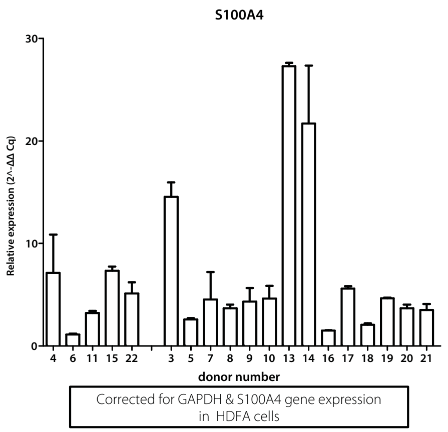
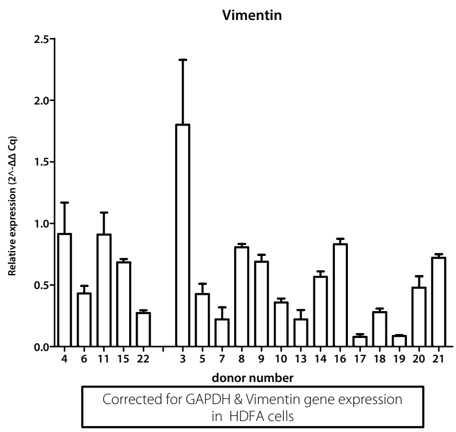


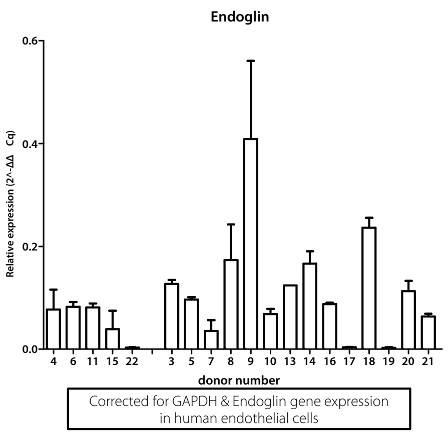
Figure 4: Analysis of differentiation of time course experiment. (A) ALP activity measured kinetically over time in cell cultures of five donors cultured in normal medium (NM) or stimulated with osteogenic medium (OM). Pictures (B) and Integrated Intensity (C) of mineralization of the matrix over time of these cultures measured using the fluorescent dye Bonetag. SaOS-2 cells and HDFA cells served as positive and negative control, respectively. Values represent mean ± SD. Bars represent 5 mm.

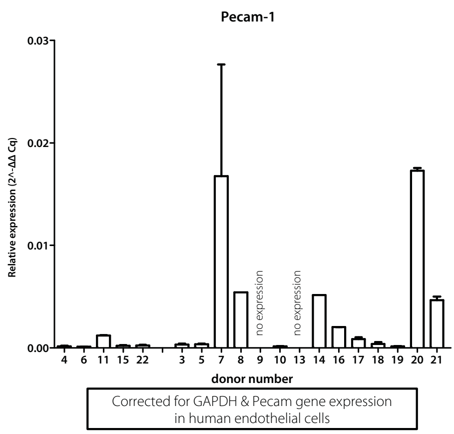

ALP
Relative expression (2^-ΔΔCq)
no expression
no expression
donor number
Corrected for GAPDH & ALP gene expression
in SaOS-2 cells


OCN
Relative expression (2^-ΔΔCq)
no expression
no expression
no expression
donor number
Corrected for GAPDH & OCN gene expression
in SaOS-2 cells


S100A4
Relative expression (2^-ΔΔ Cq)
donor number
Corrected for GAPDH & S100A4 gene expression
in HDFA cells


Vimentin
Relative expression (2^-ΔΔ Cq)
donor number
Corrected for GAPDH & Vimentin gene expression
in HDFA cells


Endoglin
Relative expression (2^-ΔΔ Cq)
donor number
Corrected for GAPDH & Endoglin gene expression
in human endothelial cells


Pecam-1
Relative expression (2^-ΔΔ Cq)
no expression
no expression
donor number
Corrected for GAPDH & Pecam gene expression
in human endothelial cells

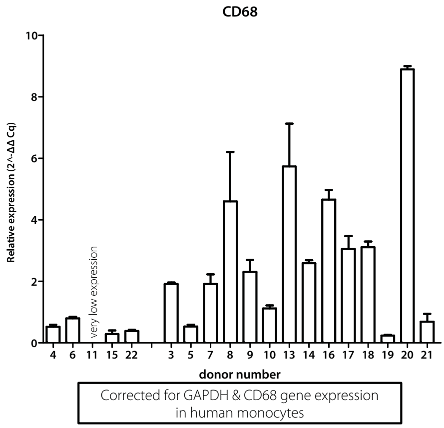


Figure 5: Relative gene expression (2^-ΔΔCq) of peri-prosthetic tissue cells. Expression of alkaline phosphatase (ALP) and osteocalcin (OCN) was corrected for internal control GAPDH and relative gene expression in SaOS-2 cells. Expression of S100A4 and vimentin was corrected for internal control GAPDH and relative gene expression in HDFA cells. Expression of Endoglin and Pecam-1 was corrected for internal control GAPDH and relative gene expression in human endothelial cells. Expression of CD68 was corrected for internal control GAPDH and relative gene expression in human monocytes.

Discussion

In the current study, we subjected peri-prosthetic tissue cells to osteogenic stimuli to investigate whether these cells could differentiate into the osteoblastic lineage. We observed an increase in alkaline phosphatase (ALP) activity and a production of a mineralized matrix upon induction/stimulation with osteogenic stimuli. Increase of ALP activity in early stages of osteoblast differentiation is assumed to reflect the number of progenitor cells committed to osteogenic differentiation in a cell population.[28] In general during differentiation, ALP first increases and then decreases when mineralization is well progressed.[28] In agreement, in peri-prosthetic tissue cultures ALP activity reached a peak at the onset of mineralization. We also observed cells stained for ALP activity after 18-21 days of culture in NM. Furthermore, in one donor, we noticed an increase in enzymatic ALP activity over time when cells were cultured in NM. In a study by Heinemann et al.[29] it was found that cells obtained from granuloma explants from endoprosthetic revisions stained positive for ALP activity, indicating that in peri-prosthetic tissue a population of cells could be present that is already committed to the osteoblastic lineage.

Mineralization of the matrix describes the final stage of osteoblast/osteogenic differentiation. Instead of using conventional staining techniques like Alizarin Red S, we have chosen Bonetag to assess the level of mineralization in our cultures, since we previously showed Bonetag to be more sensitive to small changes in mineralization.[27] In the current study, we observed an increase in mineralization when cells were cultured with OM. Moreover, since BMPs are known inducers of osteogenic differentiation[24], and a local increase of synthesis of several BMPs in the peri-prosthetic tissue has been reported[23] we also stimulated cells with OM supplemented with either BMP-2 or BMP-6. Several BMPs, including BMP-2 and BMP-6, have shown positive effects on bone formation, fracture healing and implant osseointegration in several *in vitro* and *in vivo* animal models. [30-33] BMP-2 is even used in clinical practice for accelerating healing of fractures and for spinal fusions.[30], [34] In our recent study using the murine cell-line KS483, a combination of BMP-4 and GIN (a stimulator of Wnt-signalling) was found to enhance mineralization and decrease the expression of Sclerostin (an inhibitor of bone formation) compared to BMP-4 alone.[35] Therefore, we also stimulated cells with OM supplemented with either GIN or a combination of GIN and BMP-2 or BMP-6. Our results show large inter-donor differences in response to these stimuli, ranging from no additional effect to necessity for mineralization to occur. When all data were put together no significant increase of mineralization using BMP, GIN or BMP+GIN was observed. Therefore, we were not able to ascertain a standard formula or one common pathway which guarantees osteogenic differentiation in peri-prosthetic tissue cells.

To confirm our hypothesis that there was an inter-donor degree of responsiveness to osteogenic stimuli, we performed a time-series experiment using five representative donors from the first set of experiments. Indeed, we observed an inter-donor degree of responsiveness as expressed by differences in peak-height of ALP activity and differences in time till onset of mineralization. Furthermore, we noticed an intra-donor difference in degree of mineralization at day 21 between both types of experiments. We speculate that this results from differences in cell densities or the fact that the cells were at different passages when cells were subjected to osteogenic stimuli, and therefore responses were slower or faster.

The RT-PCR results obtained in this study should be interpreted with care, since we observed variations between osteogenic and non-osteogenic conditions regarding the expression of our reference gene (data not shown). The variation could not be explained by any type of technical error. Literature, presenting RT-PCR data of peri-prosthetic tissue cells, shows the use of several different reference genes like GAPDH, 18S, β-actin, PBGD, HPRT and RPL32.[36-39] We tested all these genes, widely used as reference genes in peri-prosthetic tissue samples, but all showed differences in expression between osteogenic and non-osteogenic conditions in at least some donors. Variation in the gene expression levels of reference genes under different experimental conditions has already been found in other studies,[40, 41] indicating that it is not surprising we were not able to find the optimal reference gene for these types of cells (under these types of experimental conditions). Because of the variation in the expression level of the reference genes, calculation of cellular proportions was not possible. Therefore, in future studies, for example FACS analysis could be used to obtain data on percentages of different cell types within each sample.

In this study, we used peri-prosthetic tissue cells from early passages, since at higher passages the risk of substantial *in vitro* growth selection exists.[42, 43] To our opinion, a heterogeneous population of cells could reflect a more *in vivo*-like response to osteogenic stimuli. We tried to characterize the cell content of the tissue by investigating gene expression patterns of peri-prosthetic tissue cells at the beginning of the experiments (day 0). Since there is no suitable tissue to serve as a control for peri-prosthetic tissue, we used different control cell lines to determine the relative expression. A high variation in the gene expression levels between donors was observed, indicating heterogeneous populations of cells, which is in line with several studies evaluating the cellular content of peri-prosthetic tissue.[43-45] Although we did not find an association between the responsiveness to osteogenic stimuli and the cell content, it is not excluded that the high inter-donor variability in gene expression levels might account for the high inter-donor variability in response to osteogenic stimuli.

Besides tissue characteristics (such as cell content of the tissue), implant and/or patients characteristics, might explain some of the observed variability in the osteogenic responsiveness between the peri-prosthetic tissue of the patients. In our study, prosthesis characteristics (i.e. type of fixation) could not explain the variability in response to osteogenic stimuli. However, other studies showed that surface characteristics of implants can influence the inflammatory response to implant material and subsequently affect the expression of bone formation markers (e.g. BMP-2 and ALP).[46, 47] Therefore, in future studies, the effect of different implant materials and surface characteristics on osteogenic differentiation of peri-prosthetic tissue cells should be investigated. In addition, potentially relevant patient- and tissue-related characteristics, such as type of bone loss (linear vs. erosive) and *in situ* location of the tissue should be taken in to account. Despite our relatively large number of samples for an *in vitro* study, the sample size was still too small to perform proper association studies between different clinical groups. Therefore, in future studies, larger sample sizes of peri-prosthetic tissue with more detailed patient-, implant- and tissue characteristics might be essential to explain the inter-donor variability in response to osteogenic stimuli. As the primary aim of this study was to determine whether or not peri-prosthetic cells are capable to differentiate into the osteoblastic lineage, the experimental set-up was relatively simple. Further study is needed to determine the influence of number, size and type of particles, as well as immune cells, on the osteogenic capacity of the cells.

To our knowledge, this is the first (*in vitro*) study investigating the response of peri-prosthetic tissue cells to osteogenic stimuli. Altogether, our results indicate that these cells, cultured under osteogenic conditions, show characteristics of differentiation into the osteoblastic lineage (i.e. over time increase of ALP activity and production of a mineralized matrix), although a standard formula inducing osteogenic differentiation was not found in this study.

Acknowledgements

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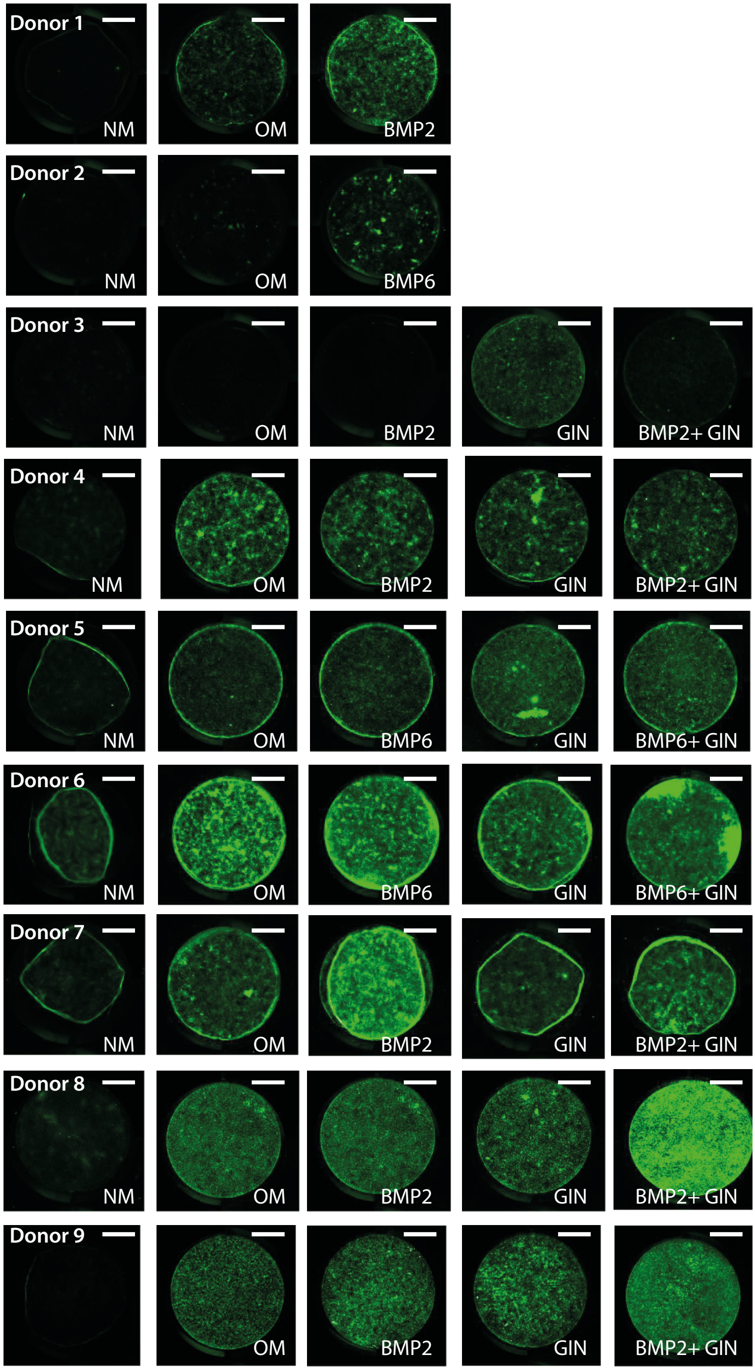
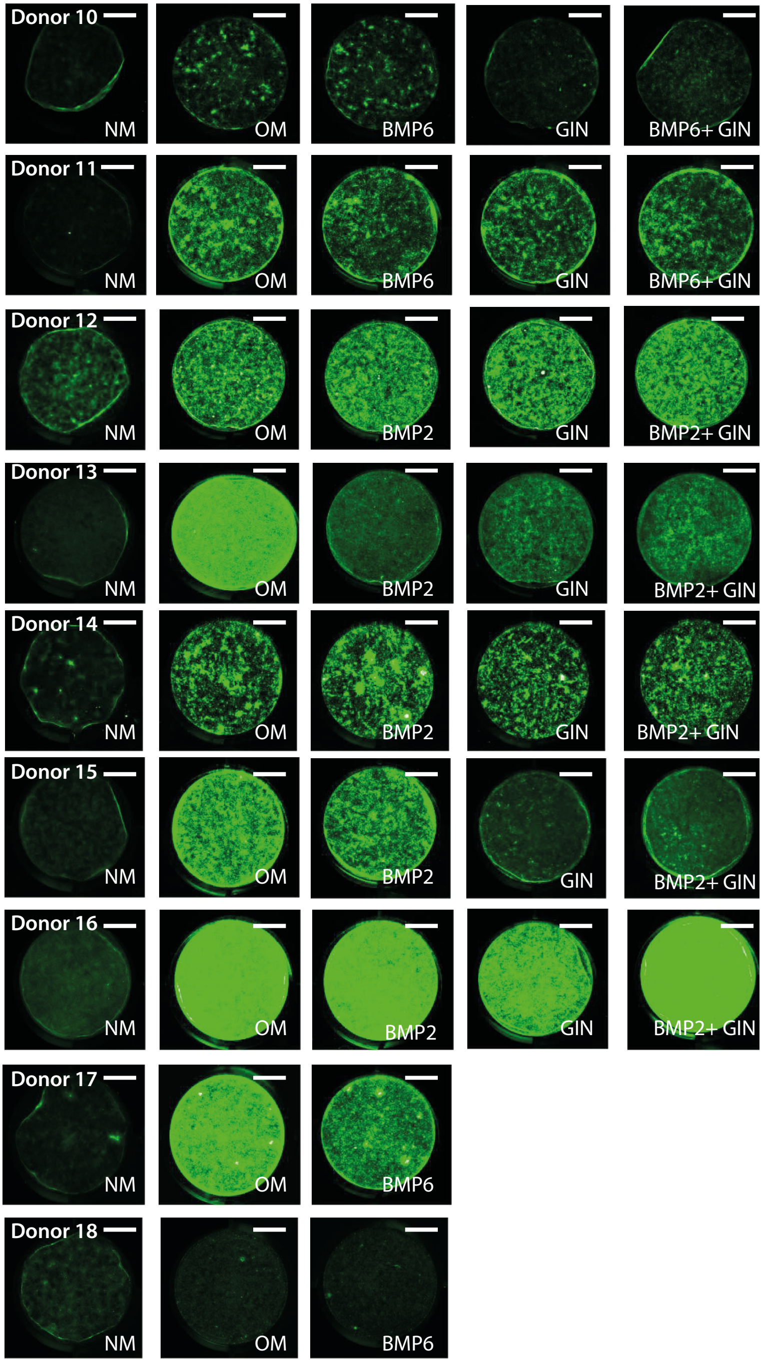
Supplementary data

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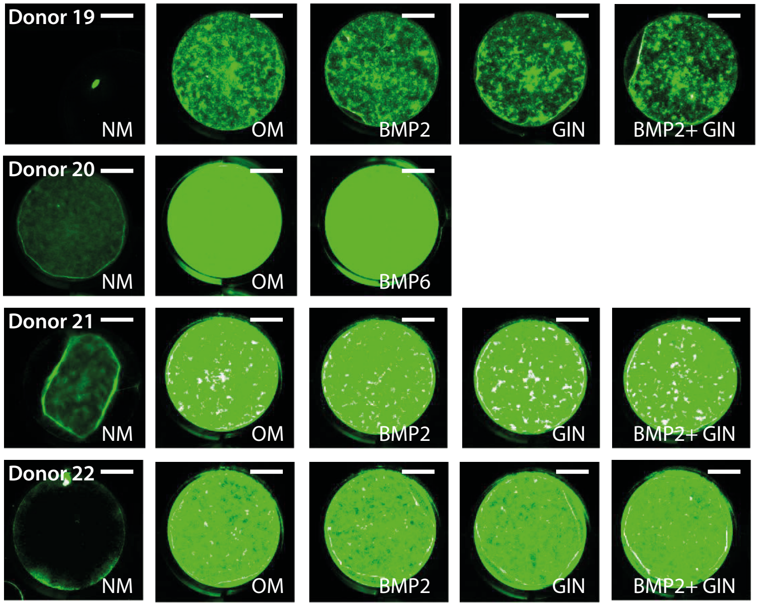

Supplementary Figure 1: Mineralization of the matrix as measured by the fluorescent dye Bonetag. Pictures of cell cultures of all donors stimulated with osteogenic medium (OM) or OM supplemented with BMP-2 or -6, GIN and BMP-2 or -6 +GIN. Normal culture medium (NM) served as a negative control. Bars represent 5 mm. BMP: bone morphogenetic protein; GIN: GSK3β inhibitor.

INNATE IMMUNE RESPONSE AND IMPLANT LOOSENING: INTERFERON GAMMA IS INVERSELY ASSOCIATED WITH EARLY MIGRATION OF TOTAL KNEE PROSTHESES

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6

Abstract

To allow prediction of the risk of loosening prior to surgery, we investigated the relationship between innate immune cytokine response via TLR2 stimulation and early migration of 6 different knee prostheses using RSA (radiostereometry). This study included 114 patients of a prospective RSA-cohort who received a total knee arthroplasty. Whole blood cytokine responses were obtained by *ex vivo* stimulation with tripalmitoyl-S-glycerylcysteine (Pam3Cys-SK4) for assessment of the TLR2 immune response. Early migration was calculated using the maximum total point motion (MTPM) one year post surgery. Principal component analysis (PCA) was applied to the cytokine data to reduce the correlated data of individual cytokines and identified 2 components. Subsequently, linear mixed model analyses were applied with adjustments for gender, age, BMI, time-to-blood sampling and prosthesis type. Component 1, consisting of IFNγ, IL-12p40, IL-10, IL-1β, TNFα and IL-6, showed a significant inverse association (β=-0.128; p=0.041) with MTPM. Further analysis showed that IFNγ (β=-0.161, p=0.008) had the highest contribution to this association and is particularly found in patients receiving another prosthesis than Nexgen (β= -0.239; p<0.001). In conclusion, patients with high levels of IFNγ upon stimulation of TLR2 are at lower risk of early migration of their knee prosthesis.

Keywords: Aseptic loosening, migration, IFNγ, TLR2

Introduction

Aseptic loosening is the most common cause for failure in total joint arthroplasty at long-term follow-up.[1,2] Mechanical as well as biological factors play an important role in the loosening process. Inadequate initial fixation and repeated strains and stresses during normal gait cycles affect the bone-implant interface.[3,4] On the other hand, loss of fixation can be caused by particle-induced osteolysis around the implant. Particulate wear debris, continuously generated by articulating motion at the bearing surfaces, is thought to trigger an aseptic inflammatory response and cause an increase in osteoclast activity and subsequent peri-prosthetic bone resorption.[5] *In vitro* studies have shown that wear particles stimulate macrophages to produce cytokines including tumor necrosis factor α (TNFα), interleukin (IL)-1β and IL-6.[6-9] Consistent with these *in vitro* findings, several clinical studies have demonstrated increased production of these cytokines in peri-prosthetic tissues and fluids of loosened prostheses.[9-11]

The mechanism of the initial cellular interaction with wear particles and the subsequent production of inflammatory mediators is still largely unknown. Recent studies suggest a critical role of Toll like receptors (TLRs) in this process.[12-14] TLRs belong to a class of receptors that enable the innate immune system to recognize foreign material and to mediate inflammatory responses.[15] It is known that different TLRs detect different pathogen associated molecular patterns (PAMPs). TLR2 mainly detects lipoproteins whereas TLR4 binds lipopolysaccharides (LPS) that are part of the membrane of gram-negative bacteria.[15] Both TLRs have been shown to be present in peri-prosthetic tissue of patients with aseptic loosening.[16] Furthermore, TLR2 has been shown to recognize wear particles and to mediate the subsequent inflammatory reaction via the TLR2 specific, MyD88 dependent signalling pathway.[12,13,17] However, a large variation in the response to wear particles both in magnitude as well as the inflammatory cytokine profile between individuals has been observed.[18] Hence, this raises the question whether diversity in clinical susceptibility to wear particles can be explained by variations in cytokine release due to differences in innate immune responses.[3] Genetic studies have already shown that associations between aseptic loosening and polymorphisms in cytokine genes exist.[19,20]

Early detection of loosening, even before symptoms occur, is possible by measuring sub millimeter migration of the prosthesis relative to the host bone.[21-23] Radiostereometric analysis (RSA) allows *in vivo* three-dimensional measurement of prosthetic migration with a high level of accuracy.[22-24] Studies have shown that increased early migration (one or two years post-surgery) is associated with increased risk of aseptic loosening and subsequent revision at the long term.[23,25] However, RSA measurements require the insertion of 1 mm tantalum in the patient's bone. Therefore, RSA can only be used to early assess loosening in patients that have been included in clinical RSA studies. Since we hypothesize that differences in innate immunity are related to the susceptibility of aseptic loosening, markers of the innate immunity might be suitable as predictors for loosening even before the patient receives joint replacement surgery.

Therefore, the aim of this study was to investigate whether there is an association between innate immunity, as reflected by the capacity to produce cytokine responses upon stimulation with the TLR2 agonist Pam3Cys-SK4, and early prosthesis migration, as measured by RSA in a cohort of patients with a total knee arthroplasty (TKA).

Material & Methods

Study design (Level of Evidence): This is a cross-sectional study (Level III).

Patients

Our cohort consisted of 137 patients who received a total knee arthroplasty (TKA) and were included previously in one of our prospective clinical RSA studies. Between February 2010 and June 2011, all patients in our cohort visited the Leiden University Medical Center for routine RSA measurements and collection of blood samples. Twenty-three patients were excluded from this study for various reasons, see Figure 1. In total 34 patients received a bilateral TKA. However, from eleven of these patients only one knee was included in the study as the other prosthesis was less than one year in situ. As a result, in the current study we have included 114 patients with 137 knee prostheses. Within this cohort, patients received different designs of knee prostheses, including Nexgen (Zimmer Inc), Triathlon (Stryker Inc), Rocc (Biomet Inc), Interax, Interax ISA, Interax PS (Howmedica Osteonics Corp).[22,26-28] The study was approved by the Medical Ethical Committee of the Leiden University Medical Center (P09.228), and informed consent was obtained from all patients.

RSA analysis

RSA radiographs were made in a uniplanar setup using a highly accurate carbon calibration box (Carbon box, Leiden, The Netherlands) positioned underneath the examination table. The first RSA examination was made before weight-bearing on the second postoperative day and served as the reference for all further examinations. All evaluations are related to the relative position of the prosthesis to the bone at that time. The RSA data was analyzed using commercially available software (Model-based RSA, version 3.34, RSAcore, LUMC, Leiden, The Netherlands) and enables determination of the relative 3D position of the markers of the prosthesis in relation to the bone markers. In situations where less than three markers could be detected, the Marker Configuration Model RSA technique was used.[29] The parameter indicating the largest three-dimensional migration of any point on the prosthesis surface is called Maximal Total Point Motion (MTPM).[24] The reason for using MTPM as measure for migration, other than translation and rotation of the prosthesis, is that motion implies a biological effect and this effect is liable to be greatest at the point of maximum motion. To measure early migration of the prosthesis, MTPM of the tibial component was assessed one year postoperatively.

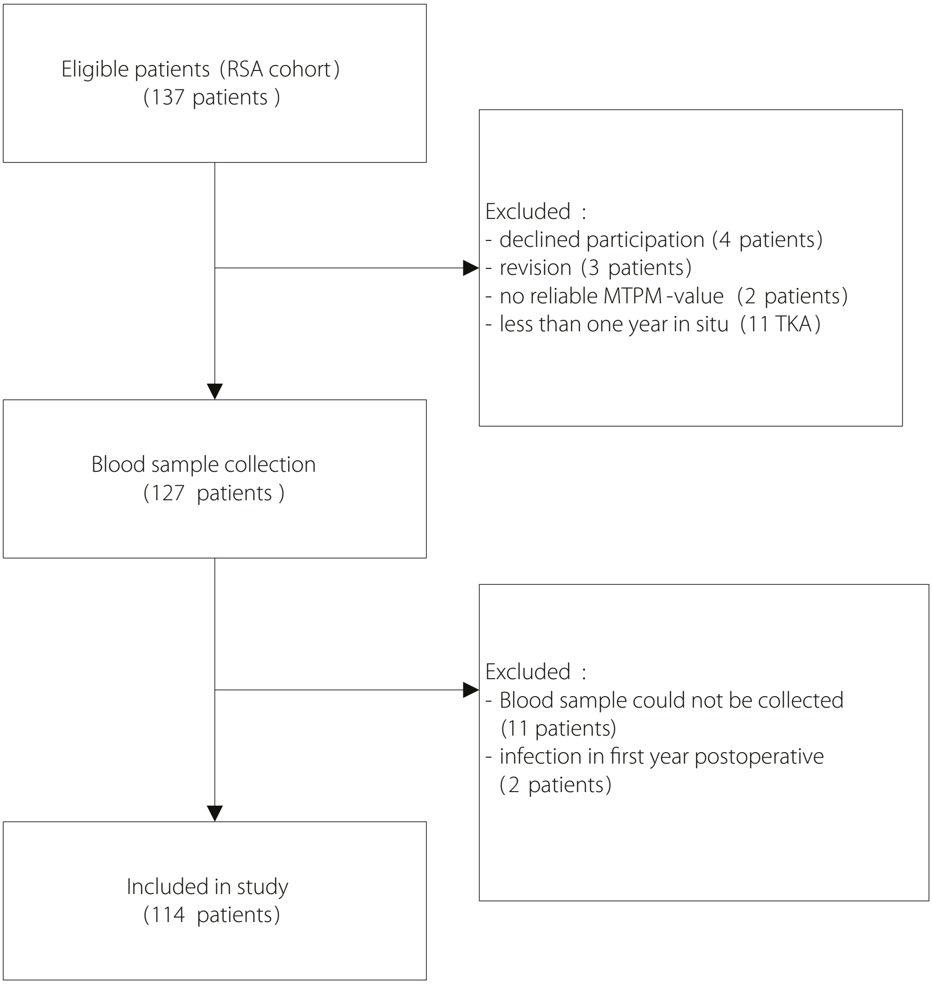

Figure 1: Flowchart of patient inclusion

Cytokine measurement

The innate immune response of patients was assessed by measuring the cytokine production capacity of whole blood samples upon *ex vivo* stimulation as described elsewhere.[30] The cytokine production capacity was assessed by *ex vivo* stimulation of 2 ml of whole blood with Pam3Cys-SK4, which stimulates the TLR2 response. Blood was collected in heparinized tubes and samples were diluted twofold with RPMI-1640 (Sigma, St. Louis, MO, USA). Samples were incubated for 24 hours with 25 µg/ml N-Palmitoyl- S-[2,3-bis(palmitoyloxy)-(2RS)-propyl]-(R)-cysteinyl-(S)-seryl-(S)- lysyl-(S)-lysyl-(S)-lysyl-(S)-lysine (EMC Microcollections, Tübingen, Germany) at 37 °C and 5% CO_2. After centrifugation, the supernatants were stored at -80 °C until assayed for IL-4, IL-13, IFNγ, GM-CSF, IL-6, TNFα, IL-12p40, IL-1ra, TGFβ, IL-10 and IL-1β using standard ELISA techniques according to manufacturers' guidelines (Central Laboratory of the Blood Transfusion Service, Amsterdam, The Netherlands). Data on IL-4, IL-13 and GM-CSF production upon stimulation was not available, since in most samples, levels of these cytokines were below the detection limit (4 pg/ml for IL-4 and IL-13; 30 pg/ml for GM-CSF).

Statistical analysis

Due to the non-normal distribution of MTPM values and cytokine levels, a log transformation was applied to these variables in all statistical models applied. A principal component analysis (PCA) was performed to reduce the multitude of correlated cytokine variables by creating 'summary' variables, so called principal components.[31] Data of IFNγ, IL-6, TNFα, IL-12p40, IL-1ra, TGFβ, IL-10 and IL-1β were entered in the PCA. Percentage of variance explained by the components and Eigen values > 1 were used to determine the number of principal components. For each variable in the extracted component(s), a "factor loading" was calculated which can be interpreted as correlation measure between the observed variable and the component. For analysis, only factor loading scores of > 0.4 (significant) qualified for loading of a variable on a component.[32]

In order to assess the relationship between early migration and the innate immune response, a multivariate generalized estimating equations (GEE) model was fitted with MTPM as dependent variable and the extracted principal components as independent variables. In all analyses, gender, age, body mass index (BMI), time-to-blood sampling and prothesis type were added as covariables. Since every knee has its own MTPM value, patients with bilateral knee replacements have two MTPM values but only one data-set of cytokine responses. To model the intrapatient MTPM correlation in patients with bilateral knee replacement, family identity numbers were included as random effect variables. Results of the GEE model analyses are expressed as estimates (β) that represent the association between MTPM and extracted principal components of cytokine levels. Statistical analyses were performed using SPSS version 20 (SPSS, Chicago, IL, USA). P-values < 0.05 were regarded as statistically significant.

Results

Baseline patient characteristics of our study are presented in Table 1. Maximal total point motion (MTPM) values of 137 knees in 114 patients were available. Median MTPM was 0.66 mm and had a range of 0.15-8.19 mm. Cytokine levels were measured from whole blood samples collected one to seventeen years postoperative. Table 2 shows the cytokine levels of whole blood samples stimulated with TLR2 agonist Pam3Cys-SK4. As cytokine responses are likely to act simultaneously in the innate immune response, high correlation between secreted cytokines (see Supplementary Table 1) was observed. To reduce the correlated data of the cytokines principal component analysis (PCA) was performed on all cytokines for which a quantitative measure was available. As shown in Table 2, two components were extracted. The first component is determined by a combination of IFNγ, IL-12p40, IL-10, IL-1β, TNFα and IL-6, and explains 53.0% of the total variation. The second component, explaining 15.4% of the total variation, is determined by a combination of TGFβ, IL-1ra and IL-10.

Next, we investigated the relationship between MTPM and the principal components extracted. We observed a significant and independent inverse association between MTPM and the first component (β= -0.128; p=0.041), however, the association with the second component (β= 0.007; p=0.911) was not significant. Respective effect sizes of covariables age, gender, BMI, time-to-blood sampling and prosthesis type are shown in Supplementary Table 2; model 1.

To explore whether the cytokine(s) of component 1 contributed equally to the association with MTPM, we performed a multivariate analysis with MTPM as dependent variable and the cytokines from component 1 as separate covariables. We found a significant inverse association with MTPM only for IFNγ (β= -0.161; p=0.008), which was independent of the other cytokine levels and age, gender, BMI, time-to-blood sampling and prosthesis type. Respective effect sizes of covariables other cytokines, age, gender, BMI, time-to-blood sampling and the prostheses types are shown in Supplementary Table 2; model 2 and 3.

Finally, it was assessed whether the association of IFNγ with MTPM showed interaction with prosthesis type. A significant interaction between IFNγ and prosthesis type was observed only for Nexgen (β= 0.337; p=0.019), independent of the covariables age, gender, time-to-blood sampling and BMI (Supplementary Table 2, model 4), indicating that the relationship between IFNγ and MTPM among patients with a Nexgen prosthesis is different when compared to patients with other prostheses types. Stratified analyses, by "Nexgen" and "other" prostheses types, subsequently showed in the "other" strata an inverse association between IFNγ and MTPM (β= -0.239; p<0.001), whereas no association was observed in the 'Nexgen" strata (β= -0.006; p=0.938), independent of the covariables age, gender, time-to-blood sampling and BMI. Exclusion of uncemented protheses, all belonging to the "other" strata, did not change the observed inverse association between IFNγ and MTPM.

Table 1: Study characteristics

		MTPM median (range)
Total number of patients (N)	114	0.66 (0.15-8.19)
Patients with bilateral prosthesis	23	
Women	90	0.64 (0.18-8.19)
Age (yrs)	66.5 ± 10.7	
BMI	27.9 ± 5.3	
Blood collection after surgery (yrs)[a]	6.6 ± 4.3	
Prosthesis type (N)		
Nexgen *(Zimmer)*	63	0.53 (0.17-5.03)
Triathlon *(Stryker Inc. corp.)*	33	0.54 (0.17-8.19)
Interax ISA *(Howmedica osteonics corp.)*	9	0.38 (0.21-1.31)
Interax PS (*Howmedica osteonics corp.)*	8	0.60 (0.15-0.73)
Interax *(Howmedica osteonics corp.)*	18	0.60 (0.19-3.40)
Rocc *(Biomet Inc.)*	6	1.27 (0.58-4.10)
Fixation method (N)		
Cemented	125	0.54 (0.15-8.19)
Non-cemented	12	0.84 (0.19-3.40)

Data are expressed as N or mean ± SD.
Data of MTPM are expressed as median (range) in mm.
[a] Mean number of years between surgery and blood sample collection.

Table 2: Principal Component Analysis of cytokines

Cytokines	Pam3Cys-SK4 (pg/ml)[a]	Component[b]	
		1	2
IFNγ	33 (40)	0.69	
TGFβ1	2737 (1432)		0.79
IL-12p40	279 (545)	0.82	
IL-1ra	16242 (10661)		0.71
IL-10	276 (432)	0.81	0.41
IL-1β	118 (180)	0.64	
TNFα	148 (446)	0.86	
IL-6	6189 (12770)	0.84	
Variance explained (%)		53.0	15.4

[a] Data are expressed as median (IQR) [b]Components with 'Eigen values' >1 are extracted after Varimax rotation with Kaiser Normalization, significant coefficients with values >0.4 are displayed.

Discussion

In the current study, the association between three-dimensional migration of knee prostheses as measured with RSA and the innate immune responses via specific stimulation of TLR2 was evaluated in a cohort of total knee arthroplasty (TKA) patients. We observed a beta of -0.128, representing an inverse association of the cytokines IFNγ, IL-12p40, IL-10, IL-1β, TNFα and IL-6, clustered in component 1 of the PCA, with respect to MTPM. Subsequent multivariate analysis showed that IFNγ independently confers this inverse effect on MTPM particularly among patients who received a prosthesis type other than Nexgen (β= -0.239; p<0.001). It should, however, be noted that the Nexgen prosthesis occurred most frequent in our cohort and had, therefore, the highest power in the interaction analyses. Since studies have shown that increased early migration expressed by increased MTPM is associated with increased loosening and revision of knee prostheses at the long term [23,25], our results indicate that low levels of IFNγ upon TLR2 stimulation could be a biomarker, able to predict such loosening particularly in patients who carry a prosthesis type other than Nexgen. Given the relative small sample size of our study, replication of our findings is necessary in larger cohorts and follow-up studies, before such a clinical application of IFNγ as biomarker becomes feasible. In this study, we did not include raw data or calculated back the respective INFg levels with MTPM values in mm because of the skewness of both the MTPM values and IFNγ values. Finding a 'beneficial' threshold level of IFNγ for clinical utility would be very desirable. However, our study should be considered an initial discovery that needs to be replicated in larger studies preferably large enough to set such a threshold.

This study is the first to indicate an inverse association between IFNγ and migration of knee prostheses. Nevertheless, the role of IFNγ in bone remodelling, which is relevant to implant fixation, has been investigated in several studies. Several *in vitro* studies showed that IFNγ either suppressed [33,34] or enhanced [35,36] osteoclastogenesis whereas another *in vitro* study showed that IFNγ positively stimulates osteoblastogenesis of human mesenchymal stem cells.[37] Furthermore, an *in vivo* study, using different mouse models of bone loss showed that the net effect of IFNγ is that of stimulating bone resorption [36], whereas administration of IFNγ increased bone formation in wild-type mice and rescued ovariectomized mice from osteoporosis.[38] Taken together, the role of IFNγ in the bone remodelling process is not completely clear, yet, based on our data, we hypothesize that IFNγ might have a beneficial effect on early migration. Whether this effect is due to either a decrease in osteoclastogenesis or an increase in osteoblastogenesis during initial fixation warrants further investigation. Furthermore, an *in vitro* study showed that titanium and polymethylmethacrylate (PMMA) bone cement particles have different effects on IFNγ signalling in osteoclast progenitor cells.[39] Therefore, it could be that the role of IFNγ in early migration differs between type of fixation. Although interesting, we were not able to compare cemented and non-cemented TKA prostheses in our data set, as less than ten percent of the measured prostheses were non-cemented. Moreover, the only non-cemented prosthesis included was the Interax prosthesis. Since the uncoated, non-cemented Interax prosthesis was shown to migrate excessively and have a three times higher revision rate compared to the cemented Interax prosthesis, we cannot exclude the role of type of fixation as possible confounder.[22,28] Nevertheless, in this dataset when we excluded the uncemented prostheses from our analyses, the observed association between IFNγ and MTPM did not change. In this study, *ex vivo* blood stimulation with the TLR2 specific agonist Pam3Cys-SK4 was used rather than stimulation with wear particles. The use of wear particles may have given a more representative biological response, however, a study by Matthews et al. has shown that cells cultured with wear particles of different materials or sizes, secrete different types of functional inflammatory mediators.[18] In this respect, Pam3Cys-SK4 responses have been shown to be easily reproduced and not to suffer from a possible contamination of endotoxins on wear particles.[13,17] Furthermore, within literature, the possible role of TLR4 signalling activated by endotoxins of cell wall of gram-negative bacteria that reside on wear particles has been frequently addressed.[12,40] For that matter, both TLR2 as TLR4 are found in tissue around loosened joint replacements.[16] Additional studies are, therefore, necessary to investigate the role of TLR4 triggered innate immune responses in early migration of prostheses, as reflected by the capacity of blood cells to produce cytokine responses upon stimulation with lipopolysaccharides. These studies may confirm the here identified inverse association of MTPM and IFNγ also via TLR4 activation.

A drawback of this study is that not all blood samples were collected exactly one year after surgery. Cytokine levels were measured from blood samples collected one to seventeen years postoperative. However, the observed association between cytokine levels, including IFNγ alone, and MTPM did not change when corrected for follow-up time as confounder. Therefore, it seems unlikely that the time to blood sampling has influenced the outcome.

Altogether our results indicate that patients with low levels of IFNγ upon stimulation of TLR2 are at higher risk of early migration of their prosthesis, particularly among patients who have received a prosthesis other than Nexgen. Eventually, our findings can be used to develop a preoperative prediction model for implant failure with specific focus on aseptic loosening. Such a prediction model will be important for patient's follow-up (i.e. the frequency of postoperative radiographic control after TKA) as well as patient assessment in the preoperative state (i.e. postponing surgery if a reasonable risk for loosening is present).

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Supplementary data

Supplementary table 1: Pearson correlations among cytokines of Pam3Cys-SK4-stimulated blood samples.

	P-value							
	IFNγ	TGFβ1	IL-12p40	IL-1ra	IL-10	IL-1β	TNFα	IL-6
IFNγ		0.721	6x10-6*	0.402	1x10-6*	2x10-3*	0x10-8*	2x10-6
TGFβ1	-0.06		9x10-4*	2x10-5*	9x10-5*	8x10-2*	1x10-4*	9x10-6*
IL-12p40	0.34	0.25		2x10-8*	1x10-32*	6x10-12*	8x10-22*	1x10-38*
IL-1ra	0.07	0.36	0.45		1x10-10*	1x10-3*	2x10-3*	5x10-6*
IL-10	0.36	0.31	0.79	0.51		1x10-8*	1x10-23*	2x10-39*
IL-1β	0.24	0.11	0.52	0.26	0.44		3x10-11*	5x10-10*
TNFα	0.50	0.28	0.68	0.25	0.70	0.50		2x10-29*
IL-6	0.36	0.33	0.83	0.38	0.84	0.47	0.76	
	Pearson correlations							

Log transformed values of cytokines were used.
*Significant at the p-values.

Supplementary table 2: Effect sizes of the linear relationship between MTPM as dependent variable and all covariates in four different models.

	Model 1		Model 2		Model 3		Model 4	
	β	p	β	p	β	p	β	p
Gender	0.058	0.744	0.095	0.577	0.067	0.684	0.115	0.485
Age	0.002	0.675	0.000	0.931	0.001	0.817	0.004	0.482
BMI	-0.008	0.525	-0.006	0.613	-0.007	0.549	-0.011	0.381
Prosthesis type								
Interax (reference)	0		0		0		0	
Nexgen	-0.133	0.670	-0.174	0.560	-0.109	0.702	-1.423	0.035*
Triathlon	-0.044	0.930	-0.174	0.727	-0.005	0.991	-0.567	0.509
Rocc	0.544	0.285	0.517	0.275	0.604	0.198	-1.430	0.218
Interax ISA	-0.311	0.338	-0.320	0.297	-0.308	0.310	-1.105	0.344
Interax PS	-0.325	0.334	-0.322	0.259	-0.287	0.305	0.180	0.742
Component 1	-0.128	0.041*						
IFNγ			-0.161	0.008*	-0.159	0.002*	-0.336	0.003*
IL-12p40			0.000	0.999				
IL-10			0.054	0.576				
IL-1β			-0.078	0.134				
TNFα			0.035	0.589				
IL-6			-0.054	0.450				
Component 2	0.007	0.911						

Supplementary table 2: Effect sizes of the linear relationship between MTPM as dependent variable and all covariates in four different models.

	Model 1		Model 2		Model 3		Model 4	
	β	p	β	p	β	p	β	p
IFNγ*Prosthesis type								
Interax (reference)							0	
Nexgen							0.337	0.019*
Triathlon							0.097	0.520
Rocc							0.530	0.052
Interax ISA							0.225	0.512
Interax PS							-0.031	0.791
Time-to-blood sampling [a]	-0.013	0.706	-0.024	0.491	-0.009	0.767	-0.030	0.385

Model 1: Relationship between MTPM and the principal components extracted.
Model 2: Relationship between MTPM and the cytokines from component 1.
Model 3: Relationship between MTPM and IFNγ.
Model 4: Relationship between MTPM and IFNγ, including an interaction term.
[a] Years between surgery and blood sample collection.
*Significant at the $p<0.05$ level.
Highlighted cells are data mentioned in the Results section.

SUMMARY AND GENERAL DISCUSSION

7

The research in this thesis represents part of a project aimed at developing a minimally invasive treatment for aseptic loosened orthopaedic implants (e.g. hip-, knee implants). In this thesis the biology of the process of aseptic loosening of implants was explored. Ultimately, these findings may lay down the foundation for the identification of biological targets to counteract the aseptic implant loosening process. Eventually a biological refixation approach for loosened orthopaedic implants may be developed. In **Chapter 1**, the process of aseptic loosening as well as aspects of the peri-prosthetic interface tissue were introduced. Aseptic loosening is the most common reason for complex implant revision surgeries, which affects on average 10-20% of total hip and knee replacement patients 10-20 years after the primary joint replacement.[1-3] During the implant loosening process a fibrous-like tissue, the so-called peri-prosthetic interface tissue, is formed at the bone-implant interface. This interface tissue usually contains a wide variety of cells, which produce cytokines and chemokines triggering bone resorption around the implant. This leads to mechanically unstable implants and ultimately a need for revision surgery due to incapacitating pain or even a peri-prosthetic fracture. Aseptic loosening of implants occurs secondary to a biological response to implant wear-debris particles, originating from the articulating joint, of which the most important cellular target is believed to be the macrophage. Nevertheless, the precise biological mechanisms underlying the loosening process have still to be elucidated. Consequently, biologic targets for successful treatment of aseptic loosening have not been found yet. Therefore, the aim of this thesis was to increase the knowledge on the biological responses around aseptic loosened prostheses by (1) characterizing the cellular content of the peri-prosthetic interface tissue, (2) studying the osteogenic potential of peri-prosthetic interface tissue cells, and (3) exploring the individual host-immune responses in relation to early migration of prostheses.

Does the cellular content of peri-prosthetic interface tissue shed new light on the mechanism of implant loosening?

Characterization of the peri-prosthetic interface tissue is one of the ways of studying the underlying mechanisms of aseptic loosening and potential therapeutic targets for treatment. Numerous studies have provided information about the cellular content of peri-prosthetic interface tissue as well as the cytokines and chemokines it produces.[4-6] In a recent review, on histological and immunological aspects of aseptic loosening, a predominance of macrophages, multinucleated cells and signs of inflammation was reported in the peri-prosthetic interface tissue.[5] However, a lack of information on the role of fibroblasts and osteocytes amongst others was noted. Since only studies based on histological evaluation (e.g. cell/tissue/organ cultures) were included in that review, important cellular and molecular mechanisms associated with aseptic implant loosening may have been missed. Therefore, in **Chapter 2**, we conducted a systematic literature search aimed at summarizing the characteristics of peri-prosthetic interface tissue based on *in vitro* findings, in order to provide an overview of the currently proposed cellular mechanisms involved in implant loosening. Besides macrophages also fibroblasts, derived from the peri-prosthetic interface tissue, were shown to be actively involved in osteoclastogenesis with subsequent pathologic bone resorption through production of inflammatory cytokines, chemokines, matrix degrading enzymes, osteoclastogenic factors and angiogenic factors. Moreover, fibroblasts were reported to be considered as potential therapeutic target in treating aseptic loosening. Again, only a few papers addressed the involvement of osteoblasts/osteogenesis in the process of implant loosening. Two papers suggested that the peri-prosthetic interface tissue of some patients with aseptic loosening exhibits osteogenic characteristics.[7, 8] Another paper showed a potentially disturbed osteogenic signalling in these patients.[9] Thus, (effects on) osteoblasts do seem to play a role in the process of aseptic loosening. Therefore, we concluded that besides the well-known role of macrophages and osteoclasts in the mechanism of loosening, the role of fibroblasts and osteoblasts should be taken into account.

These findings led us to perform a tissue characterization study of peri-prosthetic interface tissue samples collected during revision surgery for aseptic loosened hip prostheses. In that study both gene expression analysis and (immuno)histochemistry were combined to evaluate the presence of various cell types, including fibroblasts and osteoblasts (**Chapter 3**). Results showed that peri-prosthetic interface tissue samples express macrophage-, fibroblast-, osteoblast- and endothelial cell-related genes. Exploring gene expression patterns between samples revealed two components, clustering osteoblast- and fibroblast-related genes in another component than macrophage- and endothelial cell-related genes. Overall, a high inter-tissue sample variability in factor loading scores of the components was observed, which could not be explained by patient- or prosthesis characteristics. (Immuno) histochemical staining of the tissue samples showed predominance of both fibroblasts and macrophages with high inter- and intra-tissue sample variation in stained area and staining location. No significant associations were found between the stained area and patient- or prosthesis characteristics or gene expression data. Besides studying the macrophage in general, we also investigated the presences of macrophage subtypes in the tissue samples. In one-third of the samples M1(pro-inflammatory phenotype) and M2 (anti-inflammatory, pro-tissue healing phenotype) macrophages were present in comparable amounts, whereas almost two-third showed the predominance of M2 macrophages. In conclusion, fibroblasts and osteoblasts seem to be at least as important as macrophages in the aseptic loosening process. In addition, in particular M2 macrophages were present in our peri-prosthetic tissue samples.

The observed clustering of fibroblast- and osteoblast-related genes in a different component than macrophage- and endothelial cell-related genes, underscores the different roles these (clusters of) cell types have in the process of implant loosening. Furthermore, differences in component scores between tissue samples indicate that the implant loosening process as such is based on a variety of cellular mechanisms between patients but also within patients. Morowietz et al. showed that the (proportional) presence of distinct cell types within peri-prosthetic interface tissue characterize different mechanisms of loosening (i.e. wear-debris induced, inadequate initial fixation).[10] Both fibroblasts and macrophages are the prominent cell types within the peri-prosthetic interface tissue (**Chapter 3**) and can produce similar osteolytic factors in response to wear-debris particles (**Chapter 2**). However, fibroblasts might also be involved in another mechanism of loosening, where inadequate initial mechanical fixation of the implant results in an expanding fibrous tissue (like scar tissue) with subsequent micromovement of the implant. The latter preventing a close interconnection between bone and implant.[10, 11] Fibroblasts can therefore significantly influence prosthesis survival because of their inflammatory response to wear debris particles and their capacity to produce and remodel the extracellular matrix around implants.[12] The observed differences in cellular content between patients might be the reason why therapeutic strategies which (only) interfere with the osteolytic process of aseptic loosening (e.g. non-steroidal anti-inflammatory drugs (NSAIDs), antibodies to specific osteolytic mediators) [13-19] showed inconclusive results. A combined therapy, which not only targets the inflammatory response to wear debris particles and the subsequent osteolytic process but also targets the extracellular matrix production and modulation, might therefore be necessary to successful interfere with the aseptic loosening process.

Although the role of macrophages within the peri-prosthetic interface tissue has been extensively studied, recently, the role of macrophage polarization showed seemingly contradictive results. Based on retrieval and *in vitro* studies, it has been shown that the presence of macrophages of the M1 phenotype (pro-inflammatory) in the peri-prosthetic interface tissue outweighs the presence of macrophages of the M2 phenotype (anti-inflammatory, pro-tissue healing).[13] Additionally, inflammatory wear debris responses are aggravated in M1-macrophages but are suppressed in M2-macrophages.[14, 15] However, other studies reported either a predominance of M2-macrophage activation or presence of equal proportions of both macrophage subtypes. [9, 16] In our study samples, the presence of M2-macrophages outweighed the presence of the M1 phenotype, although some of our samples showed no differences between both subtypes. Koulouvaris et al. proposed that in the final stages of loosening (i.e. revision is required), the pro-inflammatory response to wear debris particles might not be decisive anymore, resulting in a more prominent presence of M2-macrophages.[9] Moreover, a local dynamic shift in the macrophage phenotype from the inflammatory M1 to the anti-inflammatory M2 phenotype is presumed a transition from a state of inflammation to tissue regeneration.[17-19] Therefore, the abundance of M2-macrophages within the peri-prosthetic interface tissue might be indicative for a regenerative capacity of this tissue and proposes the prospective use of macrophage modulation as an potential early therapeutic strategy in the loosening process. Local modulation of the macrophage phenotype, which was already pointed out and investigated by several other authors[13, 20-22], might limit the wear particle-induced inflammation with subsequent less peri-prosthetic osteolysis.

In general, a large variability in outcome measures as well as study population characteristics exists, which makes comparison between studies almost impossible. In the context of patient heterogeneity, most *in vitro* studies make use of selected cell types of animal or human origin (i.e. cell lines or specific primary cell types) to reduce heterogeneity. Although, this approach facilitates interpretation of the results, it ignores the complexity of the variety of cellular mechanisms in aseptic prosthesis loosening. As aseptic loosening involves the cross-talk between a variety of cells and the subsequent production of a wide-range of inflammatory mediators and matrix degrading factors, such basic experimental approaches are not representative of the in vivo situation. Therefore, despite the relatively high variability, more research should be focused on studying the complete peri-prosthetic interface tissue instead of selected cells, like in our study in **Chapter 3**. Another general limitation of (characterization) studies on peri-prosthetic interface tissue is the relatively small sample size. For that matter, to increase the ability to compare study results, studies with a sufficient sample size and more detailed and comparable description of study and tissue characteristics should be conducted.

Do peri-prosthetic interface tissue cells have potential osteogenic capacity?

The current strategy for the treatment of aseptic implant loosening (i.e. revision surgery) is successful with 10-year survival rates of 70-80%.[1-3, 23] However, the burden to the patient is large due to the duration of the surgical procedure, (e.g. risks for infection) and extensive blood loss causing morbidity (e.g. cardiovascular, renal) to the patient in the postoperative period.[24] Minimally invasive percutaneous implant refixation procedures have therefore been developed in order to reduce morbidity, while stabilizing the primary prosthesis with bone cement either or not in combination with eradication of the peri-prosthetic interface tissue.[25-28] Other strategies aimed at reducing the prevalence of aseptic loosening by improving the quality of the primary implant (e.g. design and material) or on interfering with the osteolytic process of aseptic loosening (e.g. the use of non-steroidal anti-inflammatory drugs (NSAIDs), bisphosphonates and antibodies to specific osteolytic mediators).[29-36] Bone resorption (osteolysis) is part of the bone remodelling process which also involves bone formation. Surprisingly, only limited attention has been paid to the role of bone formation (osteogenesis) in aseptic loosening and even less is reported on the role of osteogenesis in treatment of aseptic loosening.

Osteogenesis can be stimulated in a variety of ways, for example, growth factors can stimulate signalling pathways that are involved in osteoblast differentiation. Important osteogenic signalling pathways include the bone morphogenetic protein (BMP) and the Wingless (Wnt) signalling pathway and several studies have even shown interactions between these two pathways.[37-41] However, little is known about uncoupling these pathways and their intrinsic inhibitors (like sclerostin) and the effect on osteoblast differentiation. Since, sclerostin, acts physiologically as a downstream molecule of BMP signalling to inhibit Wnt signalling and negatively regulates bone mass [42, 43], interference with both pathways and sclerostin might prolong the effect of BMPs on osteoblast differentiation or bone formation. Therefore, in **Chapter 4**, the possibility to enhance bone regeneration by interference with BMP and Wnt signalling pathways was studied in human and murine cell lines. The interaction of both pathways on the effect on expression of SOST (the gene encoding sclerostin) was investigated using human osteosarcoma cells. Results showed that SOST expression could be either decreased with increasing Wnt signalling or increased by stimulation of BMP signalling. However, GIN (a specific inhibitor of GSK3β, which is an important part/member in Wnt signalling) could significantly decrease the BMP4-induced SOST expression and thereby uncouple BMP signalling and SOST expression. In addition, using the murine pre-osteoblastic cell line KS483, combined BMP4 and GIN stimulation could enhance osteoblast differentiation (increase both ALP activity and matrix mineralization) compared to BMP4 alone. Altogether, this study showed that uncoupling BMP signalling and SOST expression (using GIN) leads to an enhanced BMP4-induced osteoblast differentiation. This effect has potential to be used in clinical practice to induce local bone formation, e.g. osseointegration of implants or fracture healing.

Until now, stimulation of osteogenesis within the peri-prosthetic interface tissue itself has never been studied. Therefore, in **Chapter 5**, the actual capacity of peri-prosthetic interface tissue cells to differentiate into the osteoblast lineage was investigated. In addition, the potential to (further) increase osteoblast differentiation using the bone formation enhancing factors tested in **Chapter 4** was examined. Results showed that culturing peri-prosthetic interface tissue cells in osteogenic medium increased ALP staining as well as gene expression levels and resulted in production of a mineralized matrix in the majority of the donors, when compared to cells cultured in normal culture medium. In general, addition of BMPs, GIN or a combination of BMPs and GIN to the osteogenic culture medium could not significantly further increase the studied osteogenic characteristics, although in some donors it could be increased. Overall, a high inter- and intra-donor variability in response to different osteogenic stimuli was observed, which hampered the identification of a standard formula inducing osteogenic differentiation. Nonetheless, peri-prosthetic interface tissue cells were proven to possess osteogenic potential and as such stimulation of osteogenesis within the peri-prosthetic interface tissue could possibly counteract or slow down osteolysis in the aseptic loosening process. Several animal studies already showed that BMP2 can enhance neo-bone formation to fill critical-sized bone defects.[44-46] In addition, a case-report study showed that implantation of a construct with BMP2 in combination with bone marrow derived mesenchymal stem cells could fill the critical bony defect after revision surgery.[47] Furthermore, preclinical studies showed the potential of inhibiting sclerostin via a monoclonal antibody in enhancing bone formation and preventing implant loosening.[48, 49]

The ability of peri-prosthetic interface tissue to respond to osteogenic stimuli suggests that a population of cells is (already) committed to the osteoblastic lineage. As we did not find an association between the responsiveness to osteogenic stimuli and the cell content of the tissue, we were not able to pinpoint the exact cell type(s) responsible for the osteogenic capacity of peri-prosthetic interface tissue. Both our characterization study and the systematic review showed macrophages and fibroblasts to be the main cell type in peri-prosthetic interface tissue. Therefore, likely one of these cell-types, or a specific subpopulation of these cell types, is able to increase ALP production and matrix mineralization in response to osteogenic stimuli. In a study by Heinemann et al., the main cell type found in granulomas obtained from prosthetic revisions was shown to stain positive for ALP as well as CD68 (a macrophage marker).[50] In a study by Zreiqat et al, foamy macrophages but not spindle-shaped mesenchymal cells, both obtained from loosened prostheses, were shown to express and produce several osteoblastic genes and proteins.[7] On the other hand, literature also shows fibroblasts to be able to express several osteoblast related genes.[51] Interestingly, several studies have showed that specific human fibroblasts (i.e. dermal and periodontal fibroblasts) are able to differentiate into osteoblasts.[52-54] Thus, finding a population of cells within the interface tissue, which can differentiate into the osteoblastic lineage is likely to provide a new opportunity to interfere with the altered balance in bone remodelling and bone resorption in aseptic loosening.

Almost all tissue samples responded to either one of the applied osteogenic stimuli, however, there was not one single, nor a combination of factor(s) that effectively induced osteogenic differentiation in all donors. Studies investigating the osteogenic differentiation of human bone marrow derived mesenchymal stem cells also showed inter-individual variation in the osteogenic capacity of these cells.[55, 56] Moreover, in a study using adipose derived mesenchymal stem cells inter-individual variation in response to BMP2 was shown.[57] Therefore, stimulation of the osteogenic capacity of peri-prosthetic interface tissue cells warrants either further research into one universal stimulus for all patients or requires a more personalized medicine approach. In addition, further research into the influence of clinical parameters (e.g. patient- and prosthesis characteristics) on the osteogenic capacity of peri-prosthetic interface tissue cells could also be necessary to be able to develop such a universal or personalized approach.

Does the individual host immune response relate to prosthesis migration?

Cellular responses to wear debris particles play a key role in the progression of osteolysis around aseptic loosened prostheses. Phagocytosis of wear debris particles triggers the release of (pro-)inflammatory cytokines, activating pathways which lead to bone resorption. The extent of osteolysis may vary between patients due to, amongst others, differences in individual host-immune responses to wear debris, which can be related to an individual genotype.[58-60] Therefore, evaluation of differences in host-immune responses between patients could possibly help to predict the risk of aseptic loosening of the prosthesis. Currently, loosening can be detected by measuring sub millimetre migration of the prosthesis relative to the host bone using simultaneous two standard radiographs with radiostereophotogrammatric analysis (RSA).[61-64] However, RSA measurements require the insertion of 1 mm tantalum beads in the patient's bone, thus (early) assessment of loosening using RSA can only be used in patients that have been included in clinical RSA studies. Because of the genetic nature of host-immune responses it might be possible to detect the risk of loosening early, even before the patient needs joint replacement surgery. Therefore, in **Chapter 6**, the relation between innate immune responses and implant migration, using RSA, was investigated. For this purpose, whole-blood of TKA patients, with different designs of knee prostheses, was stimulated with a peptide inducing the Toll-like receptor 2 (TLR2) immune response. As a result, a variety of cytokines was produced, which were shown to cluster in two components. The component containing (primarily) pro-inflammatory cytokines correlated inversely with migration. Further analysis showed IFNγ to have the highest contribution to this association. The latter was only observed in those patients who had a prosthesis other than the Nexgen knee prosthesis, for which we have no explanation yet. In conclusion, this study shows that patients with high levels of IFNγ upon stimulation of TLR2 are at lower risk of early migration of their knee prosthesis.

Several studies have shown that progressive early migration, as measured with RSA, is associated with an increased risk at revision of knee and hip prostheses at the long term.[64, 65] Therefore, the observed association between specific cytokines and early migration of knee prostheses (**Chapter 6**), indicates that the host-immune responses might potentially be used as predictor for implant loosening. Since IFNγ showed the highest contribution to the observed association, this might be a potential "loosening" biomarker. Some studies [66-71] have reported on the role of IFNγ in bone remodelling, a continuous dynamic process, which is present around implants, leading to either implant fixation or loosening. Nevertheless, the exact role of IFNγ in these responses has not yet been fully established, since both *in vitro* studies investigating the effect of IFNγ on osteoblastogenesis or osteoclastogenesis and in vivo studies on IFNγ and bone remodelling have reported inconclusive results.[66-71] For that matter, biomarker panels instead of single biomarkers, are likely to have far greater potential to predict, diagnose and monitor the progression of peri-prosthetic osteolysis which ultimately results in aseptic implant loosening.[72] Future studies using biomarker panels might be able to identify patients with a specific cytokine profile upon stimulation of their innate immune system, which could potentially predict a patient's susceptibility to aseptic loosening. Eventually, identification of patients' specific host-immune responses to implant material could help to develop a preoperative prediction model for implant failure. This may also guide patient's follow-up moments (i.e. more frequent if at risk in order to prevent gross bone loss and subsequent peri-prosthetic fracture) as well as patient's assessment preoperatively, taking patients' specific host-immune responses into the shared decision making process for choosing for joint replacement surgery or a more conservative approach in osteoarthritis patients.

As the study in **Chapter 6** was the first study to investigate the relation between an individual innate immune response and early migration of knee prostheses, future studies have to confirm our found association. In the current study, the host-immune response was determined using stimulation of TLR2, which has been shown to be easily reproducible and not to suffer from possible contamination of endotoxins on wear debris particles.[73, 74] However, particle size or type of material influences the secreted inflammatory cytokine profile [58] and as such stimulation with wear debris particles would provide a more realistic approach investigating the association of host-immune responses and prosthesis migration. Therefore, future studies using wear debris particles should be conducted. Due to heterogeneity in patient- and prosthesis characteristics within our study, pin-pointing possible confounders in the observed association was impossible. Nevertheless, the absence of the association between IFNγ and early migration in patients receiving the Nexgen type of knee prosthesis is an indication that prosthesis characteristics might be important in the observed association. Previous studies already showed that type of fixation or prosthesis design are of influence on migration and failure rate of prostheses.[62, 75] Future association studies, with more homogenous patient and prosthesis characteristics, are therefore essential to clarify the link between individual host immune responses and prosthesis migration and ultimate implant failure due to loosening.

General conclusions and future perspectives

The findings in this thesis support the significant role of macrophages in the aseptic loosening process of orthopaedic implants. Variation regarding the presence of M1- and M2-macrophages within the peri-prosthetic interface tissue implies a potentially important contribution of macrophage polarization in the loosening process. Nevertheless, besides macrophages, the role of fibroblasts and osteoblasts in the mechanism of loosening should not be underestimated and warrants further investigation. Furthermore, the demonstrated osteogenic potential of peri-prosthetic interface tissue cells accentuates the regenerative capacity of this tissue, which provides a (new) opportunity to interfere with the altered balance (i.e. imbalance) in bone remodelling in aseptic loosening.

The large inter-tissue differences observed at all levels in all studies within this thesis, as well as described in other studies investigating the loosening process, clearly show the complexity of the loosening process. Nevertheless, this thesis provides clues for alternative therapeutic strategies to interfere with the loosening process as such and shows the potential for the use of specific host-immune responses for (early) detection and possibly prediction of loosened implants. However, further research into these directions is warranted.

General recommendations for future research are to mimic as much as possible in vivo like experimental settings, for that matter the use of the complete peri-prosthetic interface tissue, for example in organ cultures, in combination with the presence of wear particles, inflammatory cells and resorptive cells might be an option to be explored. Moreover, a sufficient sample size and detailed information on patient- and prosthesis characteristics are needed to improve the generalizability of the results.

Identification of the specific cell type(s) which possess(es) osteogenic capacity within the peri-prosthetic interface tissue might help to more specific stimulate osteogenesis. In addition, pro-osteogenic compounds other than BMP and/or GIN, like compounds influencing the Hedgehog or IGF signalling, might induce a more potent stimulation of osteogenesis of the peri-prosthetic interface tissue. Ultimately one universal osteogenic stimulus could be found, although it is more likely, to find different patient more specific osteogenic stimuli in a personalized medicine approach.

The effect of modulation of macrophage subtypes should be delineated into more detail. Converting pro-inflammatory M1-macrophages to an anti-inflammatory pro-tissue healing M2 phenotype [22, 76] might reduce the inflammatory response to wear debris particles and is thus a possible target for an early intervention in the loosening process. In this respect, specialized pro-resolving lipid mediators (SPMs)[77, 78] could be interesting to investigate, as SPMs are able to modulate the inflammatory response to biomaterials through M2 macrophage polarization.[79]

Early intervention can only be realized when loosening is detected at an early stage. With this respect, the use of biomarker panels to predict a patient's susceptibility to aseptic loosening should be studied. In addition, large prospective association studies are needed to further elucidate the link between individual host immune responses and the failure, due to aseptic loosening, of implants.[59, 80, 81]

Continuing research on the biological responses around aseptic loosened prostheses will provide a rationale for better understanding aseptic loosening of orthopaedic implants. Ultimately this knowledge provides the basis for the development of therapeutic strategies for the treatment of loosened prostheses at an earlier stage, before gross loosening due to severe osteolysis is present. In the end, this will hopefully result in the redundancy of extensive revision surgery, with high risk of morbidity to the patient might be prevented.

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APPENDIX

Nederlandse samenvatting
List of publications
Curriculum Vitae
Dankwoord

8

Peri-prothetisch interfase weefsel rondom aseptisch losgelaten prothesen: geen afval, maar een mogelijk therapeutisch doelwit?

Het onderzoek dat in dit proefschrift wordt beschreven maakt deel uit van een project om een minimaal invasieve behandeling van aseptisch loslatende orthopedische prothesen te ontwikkelen. Van alle heup- en knieprothesen faalt gemiddeld 10-20% binnen een periode van 10-20 jaar na plaatsing. De meest voorkomende reden van het falen is aseptische loslating. Aseptische loslating is een proces waarbij de verbinding tussen de prothese en het bot verzwakt raakt door een niet-infectieuze oorzaak. Tijdens dit proces wordt het bot rondom de prothese afgebroken, ook wel osteolyse genoemd. Het afbreken van bot is het gevolg van een afweerreactie, de zogenaamde immuunrespons, tegen de slijtagedeeltjes die vrijkomen van het gewrichtsoppervlak van de prothese. Het loslatingsproces gaat ook gepaard met de vorming van een littekenachtig (fibreus) weefsel, ook wel peri-prothetisch interfase weefsel genoemd. Interfase weefsel bestaat uit verschillende celtypen die, in reactie op slijtagedeeltjes, cytokines en chemokines produceren die botafbraak rondom de prothese uitlokken. Het gevolg is dat de prothese ten opzichte van het bot gaat bewegen, ook wel mechanische instabiliteit genoemd, wat gepaard gaat met pijn en een beperking van de functie van het gewricht. Uiteindelijk is het vaak noodzakelijk om een voor de patiënt belastende en technisch complexe revisie operatie uit te voeren, waarbij de prothese wordt vervangen door een nieuw exemplaar meestal samen met donorbot.

In dit proefschrift werd de biologie omtrent het aseptische loslatingsproces bestudeerd aan de hand van 3 onderzoeksvragen: (1) Kan het bestuderen van de cellulaire inhoud van peri-prothetisch interfase weefsel nieuwe inzichten geven in de mechanismes van het aseptisch loslatingsproces? (2) Hebben peri-prothetisch interfase weefsel cellen de capaciteit om bot aan te maken, de zogenaamde 'osteogenic potential'? (3) Hebben individuele immuunresponses invloed op prothese migratie? De kennis verkregen uit deze onderzoeken zal uiteindelijk bijdragen aan de ontwikkeling van een (biologische) minimaal invasieve behandeling van loslatende orthopedische implantaten.

Hoofdstuk 2 en **3** van dit proefschrift richtten zich op het karakteriseren van het peri-prothetisch interfase weefsel. In **Hoofdstuk 2** werden, middels een systematisch literatuuronderzoek, de kenmerken van het peri-prothetisch interfase weefsel (verkregen met behulp van *in vitro* studies) in kaart gebracht. Dit had als doel om een overzicht te krijgen van de huidige voorgestelde cellulaire mechanismen betrokken in het aseptische loslatingsproces. Naast macrofagen produceren ook fibroblasten inflammatoire cytokines, chemokines en enzymen die bijdragen aan de vorming en activatie van botafbrekende cellen (osteoclasten), wat uiteindelijk leidt tot osteolyse. Sommige auteurs noemen ook fibroblasten als mogelijk therapeutisch doelwit om aseptische loslating van prothesen te behandelen. Maar weinig studies onderzochten de betrokkenheid van botaanmakende cellen (osteoblasten) en het proces van botaanmaak zelf, ook wel osteogenese genoemd, in het loslatingsproces. Twee studies suggereerden dat het peri-prothetisch interfase weefsel van sommige patiënten osteogene karakteristieken bevat. Een andere studie liet zien dat de osteogene signalering in patiënten met falende prothesen mogelijk verstoord is. Allemaal aanwijzingen dat (effecten op) osteoblasten mogelijk een rol spelen in het aseptische loslatingsproces. Op basis van dit review concluderen wij dat naast de bekende rol van macrofagen en osteoclasten in het loslatingsproces, de rol van fibroblasten en osteoblasten wellicht net zo belangrijk is..

Geïntrigeerd door deze bevindingen verrichtten we een experimentele karakterisatie-studie (**Hoofdstuk 3**). Met behulp van cel-specifieke genexpressie en (immuno)histochemie werd het peri-prothetisch interfase weefsel van 47 patiënten onderzocht op de aanwezigheid van diverse celtypen, waaronder fibroblasten en osteoblasten. Resultaten lieten zien dat deze interfase weefsels macrofaag-, fibroblast-, osteoblast- en endotheel cel-gerelateerde genen tot expressie brengen. Een zogenaamde hoofdcomponentenanalyse werd gebruikt om van alle individuele cel-specifieke genen groepen van genen (componenten) te identificeren om zo gen expressie patronen tussen weefsels te bestuderen. Hieruit kwamen twee componenten naar voren, waarbij osteoblast- en fibroblast-gerelateerde genen in een ander component zaten dan macrofaag- en endotheel cel-gerelateerde genen. Over het algemeen werden er grote verschillen gevonden in de hoogte van expressie van elke component tussen de weefsels. Deze verschillen konden niet worden verklaard door patiënt- of prothese-karakteristieken. (Immuno)histochemische kleuring van de weefsels liet de overheersende aanwezigheid van zowel fibroblasten als macrofagen zien, waarbij eveneens grote variabiliteit binnen weefsels en tussen patiënten werd gezien in zowel de mate van aangekleurd oppervlak als de locatie van de kleuring. Echter, de mate van aankleuring associeerde niet significant met patiënt- of prothese-karakteristieken en ook niet met de genexpressie data. Daarnaast bestudeerden we het peri-prothetisch interfase weefsel op de aanwezigheid van macrofaag subtypes: de relatieve aanwezigheid van pro-inflammatoire M1 macrofagen en anti-inflammatoire M2 macrofagen. In bijna 2/3 van de weefsel samples was meer M2 macrofaag-aankleuring aanwezig dan M1 macrofaag aankleuring. Samengevat, heeft deze studie inzicht gegeven in mogelijke (nieuwe) cellulaire mechanismen in het aseptisch loslatingsproces, waarin fibroblasten en osteoblasten net zo belangrijk lijken te zijn als macrofagen. Daarnaast spelen met name type 2 macrofagen een rol in het eindstadium van loslating, dat wil zeggen wanneer revisie nodig is.

Osteolyse maakt deel uit van het botremodelleringsproces waar ook osteogenese deel van uit maakt. Tot nu toe hebben studies naar mogelijke therapeutische behandelingen van aseptische loslating zich primair gericht op osteolyse en is er maar weinig aandacht geweest voor de rol van osteogenese in het loslatingsproces. Daarom richtten **Hoofdstuk 4** en **5** van dit proefschrift zich op osteogenese en de stimulatie hiervan in peri-prothetisch interfase weefsel. Osteogenese kan op verschillende manieren worden beïnvloed, bijvoorbeeld door stimuleren van signaleringsroutes die belangrijk zijn voor osteogenese en differentiatie van osteoblasten, zoals de BMP en Wnt signaalroutes. Echter, door het stimuleren van deze signaleringsroutes worden tegelijkertijd remmers van osteogenese gestimuleerd, zoals bijvoorbeeld Sclerostin. In **Hoofdstuk 4** werd onderzocht of door het interfereren met BMP en Wnt signaalroutes en Sclerostin, osteoblast differentiatie en osteogenese gestimuleerd konden worden. De interactie van beide signaalroutes op de expressie van SOST, het gen dat Sclerostin codeert, werd onderzocht in een humane botkanker cellijn. De genexpressie van SOST kon ofwel verlaagd worden door stimulatie van de Wnt signaalroute ofwel verhoogd worden door stimulatie van de BMP signaalroute. Echter, GIN, een specifieke remmer van GSK3ß, dat een belangrijk onderdeel is van de Wnt signaalroute, kon de BMP-4 geïnduceerde SOST expressie significant verlagen en daarmee BMP signalering loskoppelen van SOST expressie. Daarnaast werd in de muis osteoblast-voorloper cellijn KS483 aangetoond dat in vergelijking met stimulatie met alleen BMP-4 een combinatie van BMP-4 en GIN een verhoogde osteoblast differentiatie gaf, dat wil zeggen meer alkalische fosfatase activiteit (ALP) en matrix mineralisatie. Samenvattend liet deze studie zien dat het ontkoppelen van BMP signalering en SOST expressie, met behulp van GIN, leidt tot een versterkte BMP-4 geïnduceerde osteoblast differentiatie. Dit effect kan mogelijk worden gebruikt in een klinische setting om lokaal osteogenese te stimuleren, bijvoorbeeld rondom loslatende protheses of bij fractuurgenezing. In **Hoofdstuk 5** werd de mogelijkheid om peri-prothetische interfase cellen te laten differentiëren in osteoblasten onderzocht. Daarnaast onderzochten we het in hoofdstuk 4 geobserveerde versterkende effect van BMP en GIN op osteoblast differentiatie, maar nu in peri-prothetische interfase cellen. Resultaten lieten zien dat wanneer peri-prothetische interfase cellen in celkweek werden gebracht met osteogenese stimulerend groeimedium, ALP kleuring en ALP gen expressie hoger waren in vergelijking tot celkweek met normaal groeimedium. Daarnaast produceerden peri-prothetische interfase cellen een gemineraliseerde extracellulaire matrix wanneer ze met osteogeen groeimedium in celkweek werden gebracht. Over het algemeen werden de bestudeerde osteogene eigenschappen van peri-prothetische interfase cellen niet significant versterkt door de toevoeging van BMPs, GIN of een combinatie van BMPs en GIN aan het osteogene groeimedium. Echter, in sommige weefseldonoren konden de osteogene eigenschappen wel worden versterkt of was de toevoeging van deze stoffen zelfs noodzakelijk om osteoblast differentiatie te veroorzaken. Ondanks dat er veel variabiliteit in reactie op de verschillende osteogene stimuli werd waargenomen tussen de weefseldonoren en zelfs binnen de weefsels, concludeerden we dat peri-prothetische interfase cellen kunnen differentiëren in osteoblasten. Deze studie toont daarmee een regeneratieve capaciteit van peri-prothetisch interfase weefsel aan, wat een (nieuwe) mogelijkheid biedt om in te grijpen in de verstoorde botremodellering in het aseptische prothese loslatingsproces.

Immuunresponses tegen prothese slijtagedeeltjes spelen een sleutelrol in de progressie van osteolyse rondom prothesen. Uit eerdere onderzoeken is gebleken dat de mate van osteolyse kan variëren tussen patiënten door onder andere individuele verschillen in de immuunrespons op slijtagedeeltjes. Verschillen in immuunresponses tussen patiënten kunnen daardoor mogelijk het risico op aseptische loslating van prothesen voorspellen. Meerdere studies hebben al laten zien dat progressieve migratie, wat kan worden gemeten met röntgen stereophotogrammetrische analyse (RSA), kan worden geassocieerd met een verhoogd risico op het uiteindelijk falen van heup- en knieprothesen. In **Hoofdstuk 6** onderzochten we de relatie tussen immuunresponses en de migratie van knieprothesen. Hiervoor werden bloedmonsters van patiënten met verschillende ontwerpen van knieprothesen gestimuleerd met een eiwit die de Toll-like receptor 2 (TLR2) immuunrespons opwekte. Dit resulteerde in de productie van diverse cytokines die (na uitvoeren van een hoofcomponentenanalyse) in twee verschillende groepen, ook wel componenten genoemd, bleken te clusteren. Het component wat voornamelijk pro-inflammatoire cytokines bevatte, correleerde omgekeerd met migratie. Verdere analyse liet zien dat interferon gamma (IFNγ) het hoogste aandeel had in de gevonden associatie, maar dit verband was alleen aanwezig in patiënten die bepaalde typen prothesen hadden. Daarom kan uit deze studie geconcludeerd worden dat patiënten die hoge waarden van IFNγ hadden na stimulatie van TLR2, een lager risico hebben op vroege migratie van hun knieprothese. Deze studie toont daarmee aan dat immuunresponses kunnen worden gebruikt om migratie te voorspellen. In de toekomst kunnen deze immuunresponses mogelijk gebruikt worden om een preoperatief predictiemodel voor het falen (gericht op aseptische loslating) van prothesen te ontwikkelen.

In **Hoofdstuk 7** worden alle onderzoeken van dit proefschrift samengevat en bediscussieerd en worden toekomst perspectieven besproken. Macrofagen spelen een belangrijke rol in het aseptisch loslatingsproces, waarbij variaties in de aanwezigheid van M1 en M2-macrofagen in peri-prothetisch weefsel duiden op een belangrijke invloed van verschillende macrofaag subtypes in dit proces. Daarnaast zijn fibroblasten en osteoblasten minstens zo belangrijk in het aseptische loslatingsproces. De mogelijkheid tot het differentiëren van peri-prothetische interfase cellen naar osteoblasten benadrukt de regeneratieve capaciteit van het weefsel en biedt een (nieuwe) mogelijkheid om in te grijpen in het verstoorde botremodelleringsproces rond een prothese. Echter, de grote verschillen tussen peri-prothetisch interfase weefsels gezien in o.a. weefsel karakteristieken en respons op osteogene stimuli, laten zien dat aseptische loslating een complex proces is.

Vervolgonderzoek naar de biologische reacties rondom loslatende prothesen en het bestuderen van de onderliggende mechanismen van het aseptisch loslatingsproces is daarom nodig om een nog beter beeld te krijgen van dit complexe proces. Niettemin, levert dit proefschrift aanwijzingen voor alternatieve therapeutische strategieën om in te grijpen in het loslatingsproces en laat het de potentie zien voor het gebruik van specifieke immuunresponses voor (vroege) detectie en mogelijk zelfs het voorspellen van loslatende prothesen. Hierdoor kan in de toekomst mogelijk een deel van de uitgebreide revisie chirurgie worden voorkomen.

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Curriculum Vitae

Monique Adriana Elizabeth Schoeman werd geboren op 23 juni 1988 in Wageningen en is opgegroeid in Heteren. In 2006 behaalde zij haar VWO diploma aan het Dorenweerd College in Doorwerth. In datzelfde jaar startte zij met de studie Life Science & Technology aan de Rijksuniversiteit Groningen. In 2010 behaalde zij haar Bachelordiploma en begon met haar Master Biomedical Engineering in de richting Implementatie & Functieherstel, eveneens aan de Rijksuniversiteit Groningen. Haar afstudeeronderzoek, tissue engineering van smalle bloedvaten met behulp van vetstamcellen, deed zij op de afdeling Pathologie & Medische Biologie in het UMCG in samenwerking met de afdeling Biomaterials Science and Technology, Universiteit Twente. Na haar afstudeerstage bij Nano-FM in Groningen naar de ontwikkeling van synthetische 3D celkweek producten, behaalde zij in februari 2012 haar Masterdiploma. Na het deelnemen met het Nationaal Team Lacrosse aan de Europese Kampioenschappen Lacrossse in Amsterdam in 2012 begon zij in juli 2012 als promovenda bij de afdeling Orthopedie van het LUMC onder supervisie van prof. dr. R.G.H.H. Nelissen en prof. dr. ir. E.R. Valstar. Het promotieonderzoek waarvan de resultaten zijn beschreven in dit proefschrift werd afgerond in juli 2016. Sinds 1 februari 2017 werkt Monique als function development engineer bij Koninklijke Philips in Drachten.

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